THE GENETICS OF MALARIA PARASITES: STUDIES ON PYRIMETHAMINE-RESISTANCE

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CONTENTS

SUMMARY

1. Six pyrimethamine-resistant lines were obtained by selection from 3 strains of *Plasmodium berghei yoelii* and one of *P.berghei nigeriensis*. Two of the resistant lines were isolated from cloned parasite material, while a further two were derived from U.V.irradiated parasite material.

2. An attempt was made to measure the frequency with which pyrimethamine-resistant lines appeared in the selection system.

3. The six pyrimethamine-resistant lines and the three parasite strains from which they were derived were characterised for a number of parameters.

4. The characterisation studies suggested that the pyrimethamine-resistant lines were of two types, either cross-resistant to sulphadiazine and of low dependence on PABA for growth, or sensitive to sulphadiazine and highly dependent on PABA for growth. Only one resistant line was obtained with the first type of resistance.

5. An investigation of the stability of resistance of the six pyrimethamine-resistant lines after blood and mosquito passage, and storage in liquid nitrogen gave no indication of any change in degree of resistance due to these treatments.

6. An analysis was made of a cross carried out between line 17X (pyrimethamine-sensitive and GPI-1 type) and line 33X(Pr3) (pyrimethamine-resistant and GPI-2 type). Forty product clones were derived from the product of this cross. Parasite clones with parental and recombinant combinations of characters were obtained. An attempt was made to analyse the segregation patterns of development site and PABA dependency characteristics of the product clones. Both these characters appear to segregate without the pyrimethamine-response marker during a cross.

7. An analysis was made of the response to pyrimethamine of product clones derived from a cross between the N67 (pyrimethamine-sensitive and GPI-2 type) and line 17X(Pr1) (pyrimethamine-resistant and GPI-1 type). Only two classes of product clone were obtained with respect to their response to pyrimethamine, namely a resistant and sensitive class.

8. A discussion of the selection experiments, the characterisation studies, and the crossing experiments is included. Future developments in genetic studies with malaria parasites are also considered.

INTRODUCTION

In 1948 the establishment of the World Health Organization by the United Nations made international malaria control possible. At that time the prospects for malaria eradication schemes seemed highly promising, combining mass chemotherapy with vector control. This policy has met with some success: the islands of Ceylon, Mauritius and Cyprus have eradicated malaria, while India is said to be virtually clear of the disease. However, in Africa, South East Asia and South America malaria remains a common disease. It has been estimated that current eradication programmes protect 619 million people and substantial anti-malarial operations affect a further 195 million, but 272 million people still live in malarious areas where no specific anti-malarial measures are being carried out (from W.H.O., 1971 and summarised by Peters, 1972). The failure of the eradication programme has been associated with administration problems and the growing incidence of drug-resistant strains of malaria vectors and malaria parasites.

The appearance of drug-resistant plasmodia occurred at the end of the 1940s: four years after the introduction of proguanil into the field, infections resistant to the standard drug doses were reported. The evidence for this development came from both field investigations and laboratory studies in animal and human malarias. The first field report came from Malaya (Field & Edeson, 1949) where, in 1947, mass administration of proguanil for

prophylaxis purpose, was introduced on a rubber plantation. A high level of drug-resistance in strains of P.falciparum was evident by 1949. Similar findings soon followed from Assam and Indo-china(Gilroy, 1952 and Canet, 1953).

Pyrimethamine is reputed to have been designed on the basis of its structural resemblance to cycloguanil (which had been identified as the active metabolite of proguanil), and was introduced as an antimalarial agent in the field in 1952. The first indication of the ease in which pyrimethamine-resistant strains could occur in the field came when McGregor and Smith (1952) reported that pyrimethamine in repeated doses had failed to cure a falciparum infection in the Gambia. Subsequently, Jones (1954 & 1958) reported the appearance of strains of P. falciparum and P.malariae which had become resistant to pyrimethamine in the Makueni region of Kenya. Afridi and Rahim (1962) reported the appearance of pyrimethamine-resistant P.falciparum in West Pakistan, while Maberti (1960) reported the occurrence of pyrimethamine-resistant strains of P.falciparum and P.vivax in Venezuela. Many other reports of pyrimethamine-resistant strains of P. falciparum have now come from both Asia and Africa, and these have been summarised by Peters (1970a).

These developments were not, however, considered to be too serious for malaria chemotherapy, since mepacrine and the 4-aminoquinolines such as chloroquine were thought to emulate quinine in being unable to

induce drug-resistance. This view was supported by numerous, unsuccessful attempts to induce chloroquine-resistance in various animal plasmodia, as well as one such attempt with P.vivax. Chloroquine-resistant strains, however, were reported in 1961 in Colombia with P. falciparum (Moore & Lanier, 1961, and Young & Moore 1961), and other reports from the Far East and South America have since followed. The development of chloroquine-resistant plasmodial strains was not well documented. It is now known that strains of P.falciparum vary greatly in their degree of sensitivity to chloroquine (Peters, 1970a) and it cannot be ruled out that the current situation is due to the existence of strains of P.falciparum whose sensitivity to chloroquine has always been considerably less than the more commonly encountered strains, rather than to the emergence of new resistant lines under the influence of drug use.

The increase in the incidence of drug-resistant malaria has increased the need for research into malaria. The United States of America, with its recent military involvement in South East Asia, an area where drug-resistance has become a serious problem, has undertaken a massive drug screening scheme, screening a thousand chemicals weekly. New compounds with some degree of anti-malarial activity in the test systems have been discovered, and these are undergoing or awaiting field trials (Aviado, 1969). The limited success of this screening scheme has increased the importance of research

into the development of resistance and the mechanisms underlying the action of the standard anti-malarial drugs, such as pyrimethamine, proguanil, chloroquine, amodiaquine and primaquine. It has also increased interest in studies on the biology of the malaria parasites, as these may help to locate differences in the metabolism of the host and parasite and allow a less haphazard approach to the development of new drugs or other anti-malarial measures, than occurs at the present time.

The present study uses a genetic approach to investigate pyrimethamine-resistance in rodent malaria. It is important when contemplating genetic experiments with malaria parasites to choose characters for study which are known to be stable during blood passage and mosquito transmission, and for which simple tests can be devised to distinguish between the various parasite forms because in genetic studies of malaria parasites it is necessary to analyse a large number of clones derived from the products of a cross.

Resistances to antifolates were considered particularly suitable for genetic investigation because of the reports in the literature of their stability during both blood passage and mosquito transmission. For example, Bishop and Burkett (1947 & 1948) produced a line of P.gallinaceum resistant to proguanil, by gradually increasing the drug-selection pressure over 4½ months. They reported that a sub-line was found to retain its resistance through 5 sequential mosquito transmissions

interspersed with blood passages through untreated chicks. This procedure lasted 3 months during which time the sub-line was completely free of any contact with proguanil. Subsequently, Bishop and McConnachie (1950) repeated this type of experiment on a further sub-line of the proguanil-resistant line of P.gallinaceum. They passaged this sub-line 140 times through untreated chicks doing 52 weeks without recording any loss of resistance to proguanil. Similarly, Greenberg and Bond (1954) have recorded that their pyrimethamine-resistant P.gallinaceum line maintained its resistance for at least six months while not receiving drug pressure.

This behaviour of pyrimethamine and proguanil resistant plasmodia contrasts sharply with that of chloroquine-resistant lines which have frequently proved to be unstable (Ramakrishnan et al (1957), Kollert (1963a), Jacobs (1965), Thompson et al (1965a), Peters (1965d) and Hawking (1966). Peters(1968a and 1968b) has carried out an extensive study of the loss of chloroquine-resistance during blood and mosquito passage without contact with the drug.

Resistance to antifolate drugs would also appear to be relatively easy to obtain. For example proguanil-resistant lines have been developed by a large number of workers including: Bishop and Birkett (1947), Williamson et al (1947), Knoppers (1947), Rollo (1955a) Greenberg (1949) - all in P.gallinaceum; Redmond and Fincher (1949), Grant (1950) in P.relictum; Thompson

et al (1948) in P.lophurae; Hawking and Perry (1948) and Schmidt et al (1949) in P.cynomolgi; Haswant Singh et al (1952b) in P.knowlesi, and by Rollo (1951) and Sautet et al (1959) in P.berghei. Similarly, many pyrimethamine-resistant lines have also been obtained in P.gallinaceum (Rollo, 1952a and 1952b; Jaswant Singh et al 1952a - these workers also showed that the resistance extended to the pre-erythrocytic stages; Greenberg and Bond, 1954; Bishop, 1962 - Beaudoin et al (1967) used this strain to demonstrate that resistance extended to the secondary exoerythrocytic stages; Rabinovich, 1965b and Richards, (1966b), in P.lophurae (Trager, 1961), in P.berghei (Rollo, 1952a and 1952b; Thurston, 1953; Jacobs, 1964; Rabinovich 1965a and 1965b; Vincke, 1966; Benazet and Werner, 1968 and Thompson and Bayles, 1968), in P.cynomolgi (Schmidt and Genther, 1953, and Jaswant Singh et al, 1953), and in P.knowlesi (Jaswant Singh et al, (1954a). While in human malarias, Hernandez et al (1953) were the first to report inducing experimental pyrimethamine-resistance in P.vivax and Young (1957), and Burgess and Young (1959) reported inducing this drug resistance in strains of P.malariae and P.falciparum.

It was also thought that resistance to pyrimethamine would be particularly easy to obtain as it is possible to subject large parasite populations to asingle session of drug treatments which will invariably eliminate all the drug-sensitive parasites which are present and thereby allow resistant parasites to be readily identified.

Bishop (1962) concluded that high doses of drug did not prevent the development of resistance to either proguanil or pyrimethamine, as had been suggested by Covell *et al* (1955), and in fact Diggens (1970) obtained a pyrimethamine-resistant line of *P.berghei* after subjecting infected hamsters to a daily dose of 200 mg/kgm of pyrimethamine for 4 consecutive days. Resistance to drugs such as chloroquine would appear to be more difficult to obtain by selection than resistance to the antifolate drugs. For example Thompson *et al* (1948), Schmidt *et al* (1949) Bishop and McConnachie (1952), Seaton (1951), Kollert (1963a), Hawking (1966) and Bishop (1967) have all reported some failed attempts at inducing chloroquine resistance. In addition, successful attempts have been characterised by a very slow build up of the chloroquine-resistance at least during the early blood passages under drug pressure. It should be noted however that a number of reports have been published concerning failure to induce resistance to antifolates. For example, Benazet (1964) and Bishop (1966a) reported that not all the sub-lines they exposed to cycloquanil yielded resistant lines.

Of all the resistances obtained to antifolate drugs used in malaria chemotherapy, resistance to pyrimethamine was considered of particular interest for genetic studies because of the reports of Greenberg and Trembley (1954b) and Yoeli *et al* (1969) that this resistance could be transfered. However, the evidence presented for hybrid-

ization by Greenberg & Trembley and synpholia by Yoeli et al is not considered conclusive and will be discussed in some detail later.

In addition, the reports that pyrimethamine resistance was associated with an increased requirement for PABA (Jacobs, 1964) and a decrease in the ability of the parasite lines to live in mature erythrocytes (Arnold, 1967) added extra interest to the study of this character.

A considerable amount of research concerning the biochemical basis of the action of pyrimethamine has also been carried out. A brief review of this is now presented. Pyrimethamine was developed as an anti-malarial drug during the 1950s. At that time it was already known that drugs of this type affected folic acid metabolism (reviewed by Hitchings, 1960 and 1971), although it was not known precisely how the drug caused the death of the parasite. In most organisms folic acid metabolism is concerned with the transfer of one carbon units in the synthesis of vital cell components, such as DNA bases and amino-acids (reviewed by Huennekens & Osborn 1959, and Mudd and Cantoni, 1964). Briefly, folic acid metabolism can be divided into three parts: (i) the production of tetrahydrofolic acid (FH_4) from folic acid or its component parts; (ii) the production of a large number of folate coenzymes from FH_4, and (iii) the involvement of these folate coenzymes in the

synthesis of purines, pyrimidines and amino acids.

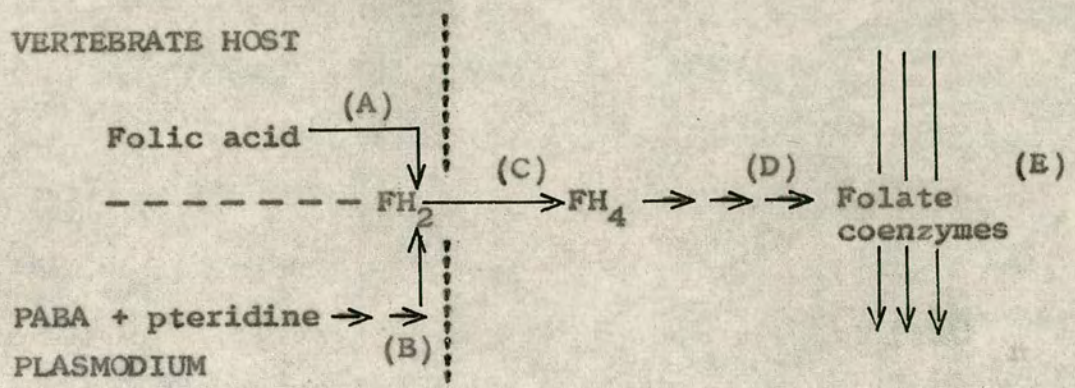
Plasmodia lack a folate reductase and are therefore unlike their vertebrate hosts in that they cannot synthesise FH_4 directly from folic acid (Ferone & Hitchings, 1966). Fig.1. illustrates the difference in FH_4 production pathways of plasmodia and higher organisms.

In an attempt to define more precisely the nature of pyrimethamine inhibition of parasite metabolism, Ferone et al (1969), isolated P.berghei dihydrofolate reductase, and demonstrated that its catalysis of the reduction of dihydrofolate to FH_4 was inhibited by pyrimethamine, and by a structurally related drug, trimethoprim. Significantly, there was a correlation of the degree of enzyme inhibition by these two drugs in vitro, and their relative effectiveness in vivo. Furthermore, they showed the parasite enzyme to be much more sensitive to pyrimethamine than the host dihydrofolate reductase. From these results Ferone, Hitchings and Burchall suggested the potent inhibition of dihydrofolate reductase to be the basis of the anti-malarial action of pyrimethamine.

At the present time there is disagreement as to the most important biochemical consequence of inhibition of this enzyme. Huennekens et al (1971), and Platzer (1972) suggest that it would prevent DNA replication by stopping the synthesis of thymidylate. In free-living organisms the synthesis of purines and some amino acids

Fig.1. Folate Coenzyme Production

(A) Folate reductase
(B) PABA + pteridine condensing system
(C) Dihydrofolate reductase
(D) Systems for the production of the folate coenzymes
(E) Involvement of the folate coenzymes in one carbon unit transfer reactions



From: Ferone & Hitching (1966), with modifications

also involves folate coenzymes. However, malaria parasites obtain these compounds from their hosts (Moulder, 1962; Polet & Conrad, 1969; Cenedella et al., 1968; Büngener & Nielsen, 1967 & 1968; Walsh & Sherman, 1968; Gutteridge & Trigg, 1970; Trigg & Gutteridge, 1971, and Platzer, 1972 - although there has been one report by Siddiqui et al., 1969, which suggested that P.knowlesi requires exogenous pyrimidines for development in vitro), and therefore the avilability of these substances to plasmodia would be unaffected by disruption in the production of folate coenzymes. In keeping with this hypothesis, Schellenberg & Coatney, (1961), found that pyrimethamine specifically inhibited the incorporation of P^{32}-phosphate into DNA.

Other workers have described results conflicting with the hypothesis that pyrimethamine prevents the synthesis of thymidylate: Aikawa & Beaudoin (1968) prepared electron micrographs of P.gallinaceum erythrocytic schizonts which had been treated with pyrimethamine. These revealed a large number of schizonts fixed at the metaphase plate stage of nuclear division. DNA synthesis occurs throughout schizont maturation and is completed by prophase within each phase nuclear division (Gutteridge & Trigg, 1970). These results suggest therefore that pyrimethamine acts highly specifically in blocking cell division after the completion of DNA replication. Furthermore, Gutteridge & Trigg (1971) observed that the inhibition by pyrimethamine of the incorporation of P^{32}-

phosphate into parasite DNA did not take place until after the time when schizonts in undrugged cultures of P.knowlesi had segmented and entered further host cells. They suggest therefore that there is some other metabolic process requiring a fully functioning dihydrofolate reductase during schizogony. Of interest to this point, Dickerman (1971) has demonstrated that the synthesis of formylated methionine required by Escherischia coli for the initiation of protein synthesis, involves folate coenzymes.

Even though pyrimethamine is known to inhibit dihydrofolate reductase activity, it is still possible that other target sites for this drug exist in plasmodia. Of interest to this point is the demonstration by Ilan et al (1969) that pyrimethamine also inhibits the P.berghei enzyme associated with the coupling of valine to a transfer RNA molecule.

In conclusion, it can be seen that we are still unable to decide how a restriction in the production of tetrahydrofolic acid, due to the action of pyrimethamine, leads to an inhibition of parasite replication. More information is required on the basic biochemistry of the parasite and in particular on how it synthesises thymidylate and what other synthetic pathways require folate coenzymes.

In view of the confusion concerning the precise nature of the anti-malarial action of pyrimethamine, it is difficult to formulate or evaluate hypotheses of the

mechanisms which might produce pyrimethamine-resistance. However, biochemical studies have been carried out on pyrimethamine-resistant P.berghei parasites (Ferone, 1969; and Ferone et al, 1970), and these suggest that dihydrofolate reductases isolated from pyrimethamine-resistant and sensitive parasite lines have differing kinetics and pyrimethamine inhibition coefficients. Diggens et al (1970) have carried out a similar study with another pyrimethamine-resistant line, and have also reported altered enzyme kinetics associated with the dihydrofolate reductase isolated from their pyrimethamine-resistant line.

The main purpose of the research programme reported in this thesis was to carry out genetic studies on pyrimethamine-resistance of plasmodia. Accordingly, a number of lines were developed which were more resistant to the action of the drug than the original parasite strains from which they were derived. During the course of this study it was suspected that further differences existed between the pyrimethamine-resistant lines. Due to the lack of resources available it was not possible to carry out a thorough examination of all aspects of these additional characters. However, an attempt was made to devise a set of simple tests which could identify clearly the differences existing between parasite lines and which would also be of use in genetic analysis. It was hoped that the study of these additional characters might facilitate the genetic analysis of pyrimethamine-resistance.

MATERIALS & METHODS

(1) Isolates, strains and lines of Plasmodium (Vinckeia) berghei Vincke and Lips 1948.

(a) Definition of terms

The application of these terms to malaria parasites in our laboratory was first defined by Carter (1971).

Isolate:- The term "isolate" is applied to parasites derived from a single wild host specimen on a unique occasion.

Strain:- The term "strain" is applied to all parasites of a single sub-species present in an isolate.

Stabilate:- Lumsden and Hardy (1965) defined the term "stabilate" as, "A population of an organism preserved in a viable condition on a unique occasion. There will be only as many examples of a stabilate as there were individual samples of it laid down on the unique occasion. It will therefore be possible to designate each stabilate by a unique code letter or number".

Line:- The term "line" is applied to parasites derived from a single strain on a specific occasion.

By strict definition every laboratory manipulation of a strain involves the creation of a new line of parasites. Thus each time blood is passaged from one infected animal to another a new line is formed. In general, however, parasites are referred to as a line

Table 1. Origins of isolates and strains of Plasmodium berghei

Sub-species of parasite	Isolate	Strain	Removal of strain from wild:- host	date	locality	History of strain	Transfer of strain to Edinburgh date	condition	References
P.b.yoelii	17X	17X	T. rutilans	April 1965	La Maboke. Central African Republic	Central African Republic ↓ Paris ≠ ↓ London (LSHTM)* ↓ Edinburgh ○	December 1967	in mouse	Landau & Chabaud (1965) - isolation from wild. Landau & Killick-Kendrick(1966)- naming of sub-species. Wéry(1968)- Life history in London (LSHTM)*
P.b.yoelii	33X	33X	"	"	"	"	"	ampoule	as for 17X
P.b.yoelii	151BY	AK	"	March 1969	"	Central African Republic ↓ Paris ≠ ↓ Edinburgh ○	March 1969	original T.rutilans captured in wild.	-
P.b. nigeriensis	N67	N67	"	August 1967	Nr Ilobi Nigeria	Nigeria ↓ London (LSHTM)* ↓ Edinburgh ○	August 1968	in mouse	Killick-Kendrick et al (1968) - isolation from wild Killick-Kendrick (1973)-naming of sub-species.

*LSHTM = London School of Hygiene and Tropical Medicine.
≠ Laboratoire de Zoologie (Vers), Muséum Nationale d'Histoire Naturelle.
○ Protozoan Genetics Unit, Edinburgh University

only after a strain has been subjected to a special form of manipulation or treatment. For instance, it is common practice to refer to a "drug-resistant line" of parasites, such a line having been derived from a drug-sensitive strain: parasites derived from a single sample of a deep frozen stabilate are usually referred to as a "line".

(b) The origins of isolates, strains and lines

Table 1 lists the isolates and strains of *Plasmodium berghei* studied in the present work. Table 2 lists the pyrimethamine-resistant lines derived in this laboratory.

I am indebted to the following for the provision of parasite material: to Professor A.G.Chabaud and Madame I.Landau for strains 17X and 33X (the stabilates held at Edinburgh were obtained from the London School of Hygiene and Tropical Medicine); to Dr R. Killick-Kendrick for strain N67 and to Mr Boulard who collected the tree rat in the Central African Republic from which strain 151BY was derived in our own laboratory.

Table 2. The pyrimethamine-resistant lines derived in this laboratory.

Pyrimethamine-resistant line	Obtained from strain	date of production
17X(Pr1)	17X	June 1970
N67(Pr2)	N67	June 1970
33X(Pr3)	33X	June 1971
33X(Pr4)	33X	June 1971
17X(Pr5)	17X	November 1971
N67(Pr6)	N67	November 1971

The pyrimethamine-resistant lines were named as follows: the name of the original sensitive strains from which the line was derived is retained, the Pr suffix denotes the pyrimethamine-resistance; the numeral indicates the order in which the various lines were obtained.

(2) Host species used in the laboratory

Table 3 lists the rodent and mosquito species used in these studies.

(3) Maintenance of malaria parasites in the laboratory.

(a) Preservation in liquid nitrogen

Stabilates of infected blood were stored in sealed capillary tubes kept in liquid nitrogen, after the method of Lumsden et al (1966). Blood infections were derived from this material by the intraperitoneal inoculation of the thawed contents of a capillary tube into a rodent.

Table 3. Host species

Host species	Strain	Breeding Type	Source
a) Rodent			
Thicket Rat (*Grammomys surdaster*)	-	Outbred	Laboratory colony
Mouse (*Mus musculus*)	C57	Inbred	Univ.of Edinburgh Centre for Laboratory Animals
	TO	Outbred	A.Tuck & Sons, Essex
	Mixed	-	Institute of Animal Genetics Mouse House colonies
b) Mosquito (*Anopheles stephensi*)			Laboratory colony

(b) Blood passage of malaria parasites in laboratory rodents.

Malaria infections were maintained in the laboratory by inoculation of blood from infected rodents into uninfected rodents. The infected blood was diluted in citrate saline (0.9% NaCl, 1.5% Na citrate, adjusted to pH 7.2) and about 0.1 ml of the diluted blood inoculated into each animal. This procedure is termed "blood passage".

Infected mice were routinely supplied with drinking water supplemented with a 0.05% p-aminobenzoic acid (PABA) to promote high parasitaemia (Hawking 1953). Mice were maintained on this dietary regime for not less than 7 days before they were inoculated with parasites. The experimental results which lead to the adoption of this level of PABA supplementation are included in Appendix A, page 123.

(c) Cyclical transmission of malaria parasites

Mosquito passage was conducted after the method of Landau and Killick-Kendrick (1966). Parasitised blood from an infection generally not more than 5 blood passages from a sporozoite-induced infection, was inoculated into a mouse or thicket rat. Four days after the inoculation the animal was placed in a cage of unfed, 7 to 14 day old Anopheles stephensi. When the mosquitoes had stopped feeding, the animal was removed from the cage. The next day a 10% solution of glucose supplemented with PABA was provided as a food source for the mosquitoes. The mosquitoes were maintained at 25°C and 90% humidity, with alternating 12 hour sequences of light and dark.

Five days after the blood feed, a sample of the mosquitoes were dissected to count the number of oocysts developed on each gut.

Ten to 14 days after the blood feed, when sporozoites were seen to be present in the salivary glands, these glands and the midguts were dissected into cold tissue culture medium consisting of modified Kitamura's medium and V.P.12 medium (Pudney and Varma 1971).

The number of mosquitoes dissected ranged between 10 and 40, depending on the level of infection recorded in the mosquitoes. The mosquito tissue was broken up with a teflon pestle and about 0.2 ml of the suspension inoculated into a rodent by the intravenous route.

Patent blood infections could be detected 4 to 6 days after the sporozoite inoculation.

(4) Red blood cell counts

These counts were carried out using a haemocytometer, on blood which had been diluted in citrate saline.

(5) "% parasitaemia" counts of infected blood.

A thin, tail blood smear was prepared, fixed in methanol and stained with Giemsa's stain. Whilst making the smear care was taken not to force blood from the tail as this can produce a sample of blood containing a higher proportion of infected cells than is present in the rodent host, due to forcing out any large parasites adhering to the walls of the tail blood vessels. In addition, counts were only made in the central area of the blood smear, as frequently the 'tail' and edges of a smear will contain a higher proportion of parasitised erythrocytes than the rest of the smear.

To standardise the errors due to sampling while estimating parasitaemias, calculations were carried out to determine the number of red blood cells to include in the sample to give a reasonable level of error at different parasitaemias. Dr Hayter of the Institute of Animal Genetics produced the following formula:-

Let n = total number of red blood cells counted

x = % parasitaemia

y = "acceptable" limits chosen

s.e.= standard error

Table 4. Red blood cell counts at different Parasitaemia levels.

Parasitaemia ± 2 s.e.	Number of RBC to be counted
0.01% ± 0.02%	10,000
0.1% ± 0.06%	10,000
1% ± 0.03%	5,000
10% ± 1.1%	3,000
20% ± 2.0%	1,600
30% ± 3.0%	1,000
60% ± 3.5%	800
70% ± 3.5%	800

Thus $x \pm 2$ s.e. $= x + \frac{x + y}{100}$

$$\text{s.e.} = \sqrt{\frac{\frac{x}{100}\left(1 - \frac{x}{100}\right)}{n}} = \frac{yx}{200}$$

$$\sqrt{n} = \frac{200}{xy}\sqrt{\frac{x}{100}\left(1 - \frac{x}{100}\right)}$$

$$n = \frac{200^2}{x^2y^2}\left(\frac{x}{100}\left(1 - \frac{x}{100}\right)\right)$$

By substituting a variety of values for x and y into the formula (i.e. chosen "acceptable" levels of error (y), at specific parasitaemias (x)), table 4 was drawn up.

The largest sample size was arbitrarily fixed at 10,000 red blood cells. The use of table 4 is illustrated by the following: if a count of a small sample of cells from a smear gave a parasitaemia of around 10%, then the original sample was extended to include an estimated 3,000 red blood cells. The erythrocytes were estimated by each microscope field in terms of 100, 150 or 200 cells, by inspection based upon much experience in actual counting.

(6) Preparation of a standardised inoculum of Malaria parasites.

Frequently experiments required large numbers of mice to be inoculated with a standard number of parasites originating from a single donor. In such cases a red blood cell count and a parasitaemia count were carried

out on a sample of the donor's blood. The subsequent preparation of the standardised inocula depended on the size of the inocula required.

(a) An inoculum size of 10^6 or 10^7 parasites per mouse:

The donor rodent was bled from the brachial vessels, the blood being collected with a pasteur pipette or syringe containing a drop of heparin. The blood was then diluted in serum Ringer (50% v/v calf serum in Ringer's solution:- KCl; 0.2gm, $CaCl_2$: 0.2gm, NaCl: 9gm, Distilled water: 1 litre, Warhurst & Folwell, 1968). The dilution step was carried out so that the required number of parasites could be inoculated in a 0.1 ml volume.

(b) An inoculum size of 1 or less parasites per mouse:

Occasionally it was required to obtain blood infections derived from a single blood parasite. After attempts at cloning by micromanipulation had proved unsuccessful, a method of "cloning by dilution" was adopted.

The procedure for the "cloning by dilution" method was as follows: a 5λ sample of blood was collected from the tail of the donor rodent and diluted in 1 ml of serum Ringer. Further dilution steps were then made until the parasite concentration was 5 to 7.5 per ml. 0.1 ml aliquots of this suspension were inoculated intravenously into a group of 20 to 30 mice. If it is

assumed that the distribution of the diluted parasitized erythrocytes to the inoculated mice conforms to a Poisson distribution, then it follows that if one in three (or less) of the inoculated mice become infected, on average 80% (or more) of these infections will have originated from one parasitized erythrocyte (Carter: unpublished information). To reduce the possibility of infections arising from doubly infected erythrocytes, donor animals were chosen with very low parasitaemias, for in these double infection of red blood cells is rare. If more than one third of the mice became infected it was probable that the inocculum contained more than on average one parasite and the experiment was discarded.

(7) The source, preparation and administration of drugs:

(a) Pyrimethamine

a) Source: Pyrimethamine (Daraprim, 2, 4 diamino-5-p-chlorophenyl-6-ethyl-pyrimidine) in the form of powdered base was obtained from the Wellcome Research Laboratories, England by courtesy of Dr. R.A.Neal.

b) Calculation of drug dose: drug dose was related to the mean weight of a group of experimental mice. The unit of dose was mg/kgm body weight. These calculations were made in order that a given amount of drug could be injected in a 0.1 ml volume.

c) The preparation and administration of the drug suspensions: Two methods were used and it will be

indicated in other sections of this thesis which of these methods was used during a particular experiment. The methods were as follows:-

(i) The method of Yoeli et al (1969). A suspension of 250mg/ml of pyrimethamine in distilled water was made, to this was added one drop of Tween 80 per 10 mls of suspension. The suspension was sonicated for 5 minutes. Further dilution from this stock was made with a 2% solution of sodium carboxymethyl cellulose. Drugs were thoroughly shaken on a vortex mixer immediately prior to their administration to mice.

This preparation was given to mice by the subcutaneous route. Control mice were given carboxymethyl cellulose.

(ii) After receiving advice from Dr. R.A.Neal, of the Wellcome Research Laboratories, pyrimethamine was administered by the oral route in view of the more regular and complete absorption of the drug by this route. The method used for preparing solutions of the drug for this route of administration was as follows: the quantity of pyrimethamine required was weighed and diluted with a weak solution of acetic acid, and a 2% solution of carboxymethyl cellulose. The ratio of volumes of these two solutions was 1:5. Drugs were thoroughly shaken on a vortex mixer before administration to mice.

Control mice were given acetic acid with carboxymethyl cellulose.

(b) Sulphadiazine

a) Source: Sulphadiazine (2-sulphonamido-pyrimidine) in the form of a dry powder was obtained from May & Baker, England.

b) Calculation of drug dose: As with pyrimethamine, doses of sulphadiazine were related to the mean weight of the experimental mice to be injected.

c) The preparation and administration of sulphadiazine. A stock solution of sulphadiazine at a concentration of 20 mg/ml was prepared by dissolving the powder in 1N NaOH and adjusting the pH to 9.6 - 9.8 with 1N HCl. Further dilution from this solution was made with NaCl at pH 9.6.

d) The diet of experimental mice used in sulphadiazine studies: Sulphadiazine is antagonised by PABA, therefore when mice were required for sulphadiazine studies, their drinking water was supplemented with only a 0.005% solution of PABA (instead of the standard laboratory regime of a 0.05% PABA solution). This dietary regime was started when the mice arrived in the laboratory, and all mice were maintained on this level of supplement for at least 7 days before being inoculated with parasites.

An evaluation of the use of this dietary regime in conjunction with sulphadiazine is given in a later part of this thesis, page 102.

(8) The selection of pyrimethamine-resistant lines of P.berghei.

The parasite material for these experiments was of three types: a) normal, untreated parasites derived from a wild rodent and thereafter maintained by blood and cyclical passage; b) parasites recently cloned by dilution; and c) parasites which had been UV. treated.

A "single step" method similar to that used by Diggens (1970) was adopted. Pyrimethamine-sensitive parasites were harvested from 2 to 12 mice. The parasites were diluted in serum Ringer and inoculated into 10-60 mice by the intraperitoneal route. When the parasite inoculations had been completed an estimate was made of the number of parasites inoculated per mouse, by carrying out red blood cell and parasitaemia counts on the diluted blood.

Pyrimethamine was given for four consecutive days commencing 0-8 days after the parasite inoculations. Daily doses of between 50 and 200 mg/kgm were given. The drug was administered sometimes by the subcutaneous and sometimes by the oral route. The route of administration in individual experiments has been specified in Table 6, in the Results Section.

An estimate of the mean number of parasites present per mouse on the day of commencement of drug treatment was calculated from: a) the mean parasitaemia taken from the blood smears of a sample of five mice: b) a red blood

cell count of the mouse whose parasitaemia was closest to the mean value obtained for the group, and c) the mean blood volume (the estimate of this latter value ranged from 3 to 5 mls and depended on the size of the infected mice).

If the parasite inoculations and the first drug injection occurred on the same day, the number of parasites inoculated per mouse was taken as the estimate of the number of parasites present at the start of the drug treatment.

The selection experiments were followed by examining individual blood smears, 8, 12 and 21 days after the completion of the drug injections. Mice which were infected on days 8 or 12 were sacrificed and a suppressive test carried out with the harvested parasites, in fresh mice.

(9) UV irradiation of blood-form malaria parasites

The UV source was a Phillips T.U.V., 15 watt, medium-pressure, mercury-vapour lamp, 90% of its output being at 2537 Å. Doses were measured using a "Jagger Meter". The dose rate was 28 ergs/mm^2/sec, variation in dose being obtained by altering the UV exposure period.

Parasites to be irradiated were collected from the brachial vessels of a group of well-infected mice and suspended in citrate saline. A brown layer parasite concentrate was then obtained (p. 36) which was

resuspended in 30 mls of phosphate saline, pH 7.4 with glucose (Krebs & Eggleston, 1940). This solution was made up in the following way: 52.5 mls of Krebs solution. (50 mls, 1.8% NaCl; 2 mls 3.0% KCl ; 0.5 mls, 7.68% $MgSO_4.7H_2O$), 52.5 mls of a 10% Glucose solution, 30 mls of a phosphate buffer pH 7.4 (24.3 mls, 0.2M , Na_2HPO_4; 5.7 mls 0.2M NaH_2PO_4). Aliquots of this suspension were kept on ice.

(a) The production of a UV. dose-response curve

It was necessary to obtain a dose response curve of UV. dose against parasite viability, before subjecting large numbers of parasites to UV. irradiations.

The aliquots of the concentrated parasite suspension were given varying doses of UV. ranging from 0 to 7,000 ergs/mm^2. During the irradiation period the parasites were kept in shallow, iced petri dishes and were constantly stirred. Parasite viability was measured using the modified Warhurst bioassay ($P._{33}$). For this test identical numbers of parasites from each aliquot were inoculated into four mice. The mean length of time was recorded for the mice in each group to exhibit a 1% parasitaemia. It was assumed that this time interval was directly proportional to the number of viable parasites originally inoculated, and therefore an estimate was possible of the number of parasites which had been eliminated by each UV. treatment. It was assumed that UV. acts by killing a percentage of

parasites rather than causing a period of pre-patent slowed growth.

The dose-response curve obtained is included in the Results section (p. 49).

(b) UV irradiation of blood-form parasites prior to selection experiments.

For pyrimethamine selection experiments, the aliquots of concentrated parasite suspensions all received 5,000 ergs/mm^2 of UV. The conditions of the parasites during the irradiation period were the same as those of the parasites used in the production of the UV dose-response curve.

After UV treatment, the parasites were pooled and inoculated into mice. The time interval between harvesting the parasites and the inoculation of the irradiated parasite population into experimental mice was 2½ hours. Thereafter the experiments followed the course of the standard pyrimethamine selection experiments. Several days were allowed to elapse before the commencement of drug treatment.

(10) Drug tests of the blood-form of malaria parasite

A number of drug tests were investigated in this study. The primary aim was to develop a test to differentiate between the responses of a number of recently isolated P.berghei strains and lines, which would balance the need for a precise and repeatable test

against the necessity in genetic work of a simple and quick test to allow a large number of lines to be tested by one person.

Peters (1970a) has drawn up a list of the factors which must be standardised in an *in vivo* drug test on the blood forms of rodent malaria. These are as follows-

a) mouse strain

b) sex and age of mice

c) concomitant infections in mice - the effects of *Eperythrozoon coccoides* and *Haemobartonella muris* on the course of plasmodium infections has been well documented(Kretschmar, 1963; Peters 1963 & 1965a; Ott and Stauber, 1967, and Büngener, 1968).

d) environment - in relation to temperature, stress and PABA levels.

f) time required for preparing inocula

g) route of administration of drug

h) timing of first drug dose

i) frequency and duration of drug administration

j) timing of termination of experiments

k) technique of reading blood films.

These factors were controlled, as far as possible during drug tests. In addition it was found necessary to standardise the age of infection of the donor mouse - a four day infection being used.

In all the drug tests undertaken, particular care was given in standardising: a) the time interval between

Table 5. The simple suppressive test: the procedure for testing 2 parasite lines, A and B, with pyrimethamine

Procedure	Line A		Line B		Time
Inoculation of 10^7 Parasites/mouse	4 mice		4 mice		Day 0
1st pyrimethamine Injection	2 mice (no drug)	2 mice (15mg/kgm)	2 mice (no drug)	2 mice (15mg/kgm)	Day 0 + 1-2 hours
2nd "	"	"	"	"	Day 1 + 1-2 hours
3rd "	"	"	"	"	Day 2 + 1-2 hours
4th "	"	"	"	"	Day 3 + 1-2 hours
Tail blood smear from all mice					Day 4 + 1-2 hours
Presence of parasites in smear	+	-	+	+	
Classification of line	drug-sensitive		drug-resistant		

the bleeding of the donor mice and inoculating the recipients; b) the time interval between the inoculation of parasites and the first injection of drug; c) the randomising of the mice by weight throughout the experimental group of mice, and d) the laboratory conditions of the experimental mice. The two types of drug test employed in this study were as follows:

(a) The simple suppressive test

This test was primarily used for the analysis of the products derived from crosses carried out between parasite lines. The test allowed a line to be classified as either drug-sensitive or drug-resistant.

The procedure for the test is summarised in Table 5. From this table it will be seen that the presence of parasites in the blood of drug treated and undrugged mice on day four resulted in the parasite line being classified as drug-resistant, whereas if only the two mice which did not receive drug treatment exhibited parasitaemia on day four, the line was described as drug-sensitive.

(b) The modified Warhurst bioassay

The Warhurst bioassay (Warhurst & Folwell, 1968) is a complicated test requiring the use of a number of parasite inocula of different sizes. Peters (1968a), described a simpler form of the bioassay involving one size of inoculum, which is only valid if all the

parasite lines being compared for their response to a drug grow at equal rates during the test period, both in the presence and absence of the drug.

The test measures the effect of a single injection of the drug on the time interval between inoculation of blood parasites into a mouse and the presence of a certain level of erythrocyte invasion in the mouse. This time interval is here termed the "test period". As this study included P.b.yoelii lines, the end-point was taken as a 1% parasitaemia. At this point in a blood infection of P.b.yoelii growth is still exponential.

The procedure followed for testing one parasite line was to inoculate 4-6 week old C57 or T.O. mice with 10^6 parasites. After three hours, each mouse received an injection of drug or injecting medium. Each dose of drug was given to 4 mice. Blood smears were prepared daily for each mouse, commencing 24 hours after the drug injection, and these were terminated after a 1% parasitaemia was recorded for each mouse. The course of infection in each mouse was examined by plotting the log % parasitaemia against the day post inoculum. The average duration of the "test period" was calculated for each group of mice receiving a different drug treatment.

So that tests carried out on separate occasions could be directly compared, the effect of the drug treatment was also expressed as the increase in the length of the "test period". This increase was calculated by

subtracting the average length of the "test period" recorded for the mice not receiving the drug from the average duration of the "test period" recorded for a group of mice receiving a certain dose of drug.

Drug doses used

(i) pyrimethamine - At the beginning of the research, trials were carried out using various drug doses. Eventually drug treatments of 2 and 50 mg/kgm were selected as the most suitable, because 2 mg/kgm was found to be sufficient to delay the drug sensitive parasite lines, 17X, N67 and 33X in reaching a 1% parasitaemia, whereas 50 mg/kgm was found to affect the time taken by the selected 'resistant' lines in reaching the end point. At a later date a 75 or 100 mg/kgm dose level was introduced as it was hoped that these larger doses would differentiate between the levels of resistance exhibited by the 6 pyrimethamine-resistant lines obtained by selection. All drug treatments in these tests were given by the oral route.

(ii) Sulphadiazine - As a range of 3 drug doses with the Modified Warhurst Test proved to be successful with pyrimethamine, it was decided to use the same procedure with sulphadiazine drug tests. It was not considered necessary to prepare a dose response curve for this drug as the most suitable highest drug dose was known to be one which delayed the growth of the parasite

inoculum in reaching a 1% parasitaemia, but did not delay it to such an extent that the immune responses of the host would be brought into action and affect the growth rate of the parasites. The first tests using 500mg/kgm as the highest drug treatment recorded delays of between one and five days for different parasite lines and therefore suggested that this dose was sufficient to distinguish between the responses of the parasite lines to sulphadiazine. The other drug treatments given were 25 and 125 mg/kgm.

(11) Starch gel electrophoresis for enzyme typing

The presence of glucose phosphate isomerase variants in blood parasites was detected by starch gel electrophoresis as described by Carter (1970). Parasitised blood was collected from rodents by bleeding from the brachial vessels into citrate saline. After centrifugation at 1,000 g for five minutes, the upper brown layer of parasitised cells was removed and freeze-dried to break up the parasites. For electrophoresis, 2 mg of freeze-dried material was dissolved in 0.02 mls of distilled water. Filter paper strips were soaked in the extract samples for insertion into the gel.

The gel buffer was 0.01M Tris HCl pH8.0 and the electrode buffer 0.05M Tris HCl pH 8.0. The regions of enzyme activity were made visible by linking the reaction to the reduction of the dye MTT tetrazolium,

which formed an insoluble blue deposit. On this system, the parasite GPI enzymes could be clearly distinguished from host blood enzymes, as the former moved towards the anode, while the latter moved in the cathodal direction.

(12) The test of PABA requirement for growth of parasite lines.

A simple test was adopted. Parasites were harvested from mice maintained on the normal 0.05% PABA water supplement. 10^6 parasites were inoculated into 8 male T.O. weanlings. Four of the mice were being maintained with the 0.05% PABA supplement, while the remaining mice had never been given PABA.

On day 4 post-inoculum, individual blood smears were taken and the ratio of mean parasitaemias of the two groups of four mice determined. This ratio was used as the measure of the PABA requirement for growth of the parasite line.

(13) The classification of the parasite line according to the development site in the rodent host.

The parasite lines could be distinguished by their development in different types of erythrocytes. Two classes of line were observed: a) those which mainly developed in reticulocytes, termed 'R' lines, and b) those which developed in both mature erythrocytes and reticulocytes, termed 'M & R' lines.

At low parasitaemias, Giemsa-stained blood-smears

of both classes of parasite line appeared identical. It was customary, therefore, to wait until a 10% parasitaemia was present before describing the development site of a line.

The classification of development site of a parasite line was carried out with T.O. or C57 mice which had been injected with 10^6 parasites.

(14) Genetics

Only the results obtained with one cross are reported in detail. The procedure for conducting and analysing the cross was undertaken in five stages: a) conducting the cross and recovering the products of the cross; b) testing for reassortment of the parental characters among the products; c) cloning the products; d) characterisation of the cloned products and (e) control studies.

Details of the procedure adopted for each stage

(a) Conducting the cross and recovering the products

The general method for conducting a cross has been reported elsewhere (Walliker et al., 1971 and 1973) and is summarised in Figs 2 and 3. Groups of rodents were inoculated with one of the two parasite lines. On the fourth day post-inoculum, the rodent infections were examined for exflagellation and the number of exflagellating bodies observed per microscope field recorded. Two rodents, one infected with each parasite line, were

Fig. 2 The procedure adopted in making a cross between two lines of P.berghei, with concomitant transmission of the pure lines.

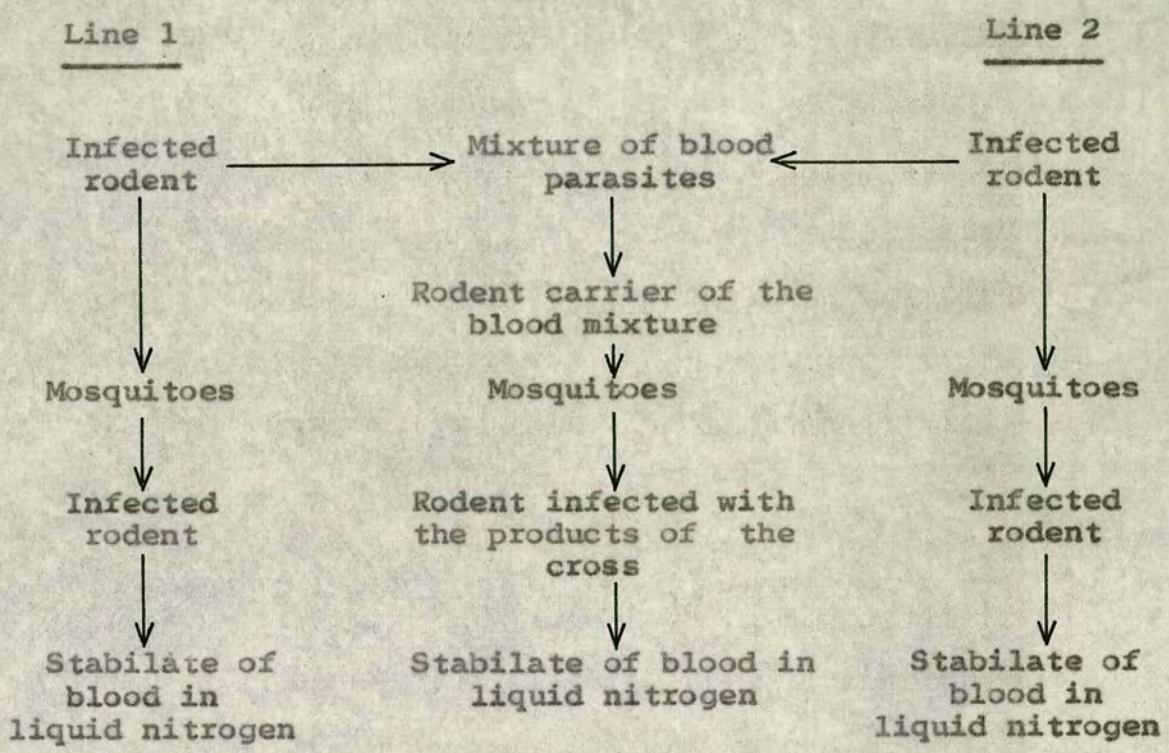
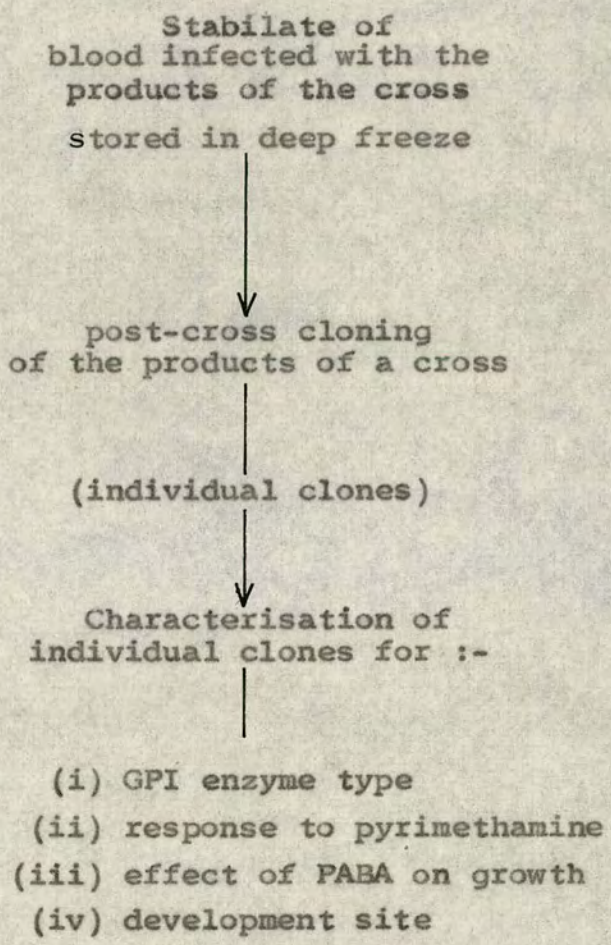


Fig. 3 The procedure adopted for the analysis of the products of a cross



chosen whose infection showed approximately equal exflagellation counts.

The rodents were bled using heparin to prevent clotting (5 iu/ml blood). The blood was mixed and inoculated intravenously into an uninfected rodent. An immediate patent parasitaemia was produced. The rodent was placed in a cage of mosquitoes.

After 5 days, the mosquitoes were examined for oocysts. Sporozoites were harvested on the thirteenth day and inoculated into thicket rats. Patent blood infections were normally observed in these rats 4-8 days after sporozoite inoculation. A sample of this infected blood was stored in liquid nitrogen.

(b) Testing to demonstrate reassortment of the parental characters among the products of a cross.

A simple suppressive drug test was carried out on the uncloned products of the cross between pyrimethamine-sensitive and resistant lines. A daily drug dose of 15 mg/kgm pyrimethamine was given. On the fourth day post-inoculum, all the mice were sacrificed and their blood individually analysed for enzyme type (p. 36)

The object of this test was to see if the GPI type originally associated with the pyrimethamine-sensitive parental parasite line was present among the products of the cross surviving the drug treatment.

Parallel simple suppressive tests were carried out on pure line parasite material, to ensure that the drug regime used would eliminate only the pyrimethamine-

sensitive parasites.

(c) Cloning of the products of a cross.

Cloning was carried out by dilution, using the method described in a previous section (p. 23).

(d) Characterisation of the cloned products

The pure lines involved in the cross described in detail, in this study, differed in four characters, namely: (i) GPI type; (ii) response to pyrimethamine; (iii) the effect of PABA on growth, and (iv) development site.

The clones derived from the products of the cross were analysed with respect to these characters. This was carried out using the tests devised to study the pure line parasites, except that in the case of (iii) only 3 mice were assigned to each PABA level.

(e) Control studies

The parasite lines involved in the cross were concomitantly mosquito passaged with the blood-mixture infection. Thereafter the pure line parasite material received the same treatment as the products of the cross.

Table 6 The Pyrimethamine selection experiments

Exp. No.	Sensitive parasite line	Treatment of parasite material	No. of mice inoculated	No. of parasites inoculated per mouse	Mean No. of parasites per mouse at the time of the first drug injection	No. of days between the parasite inoculation and the first drug injection.	Daily drug dose (mg/kgm) *	Designation of resistant line obtained
1	N67	-	22	10^7	10^7	0	200 (s)	-
2	"	-	12	10^7	10^7	0	200 (s)	-
3	"	-	17	10^8	10^8	3	200 (s)	-
4	"	-	30	10^7	10^8	3	150 (s)	-
5	"	-	13	10^7	10^9	5	100 (s)	N67(Pr2)
6	"	Cloned	30	10^7	10^9	7	50 (o)	N67(Pr6)
7	"	Cloned	16	10^7	10^9	7	50 (o)	-
8	17X	-	18	10^7	10^7	0	200 (s)	-
9	"	-	21	10^8	10^7	0	200 (s)	-
10	"	-	25	10^7	10^9	8	50 (s)	17X(Pr1)
11	"	-	30	10^6	10^7	7	50 (o)	-
12	"	UVirradiated	30	10^6	10^7	7	50 (o)	-
13	"	Cloned	50	10^7	10^8	3	50 (o)	-
14	"	Cloned	19	10^7	10^9	6	50 (o)	17X(Pr5)
15	"	Cloned	57	10^7	10^8	6	50 (o)	-
16	33X	-	30	10^7	10^8	6	100 (o)	-
17	"	-	13	10^7	10^8	4	150 (o)	-
18	"	-	18	10^7	10^9	5	50 (o)	-
19	"	UVirradiated	17	10^7	10^9	5	50 (o)	-
20	"	UVirradiated	30	10^8	10^9	8	50 (o)	33X(Pr3)+33X(Pr4)
21	"	Cloned	50	10^7	10^9	6	50 (o)	-
22	151BY	-	45	10^7	10^8	6	100 (s)	-
23	"	-	23	10^6	10^8	8	100 (s)	-

*the route of administration of the drug is indicated by the following:

(s) - subcutaneous, and (o) - oral route.

RESULTS

Section I : The development of pyrimethamine-resistant lines of P.b.yoelii and P.berghei nigeriensis.

Variations in the procedure of the selection experiments.

The four strains of pyrimethamine-sensitive plasmodia used in this study were, P.b.yoelii 17X, 33X and 151BY, and P.berghei nigeriensis N67 strain.

In a number of experiments the parasite material received special treatment before drug selection. In six experiments the parasite material had previously been cloned. In three experiments the parasites were subjected to U.V. irradiations several days prior to the start of the drug injections.

In most experiments a daily drug dose of 100 or 50 mg/kgm of pyrimethamine was given, by the oral route.

Presentation of results of the selection experiments

Parasites surviving the drug selection pressure were tested by the simple suppressive drug test. The line was termed resistant if the drug test showed it to be more resistant to pyrimethamine than the sensitive line from which it was derived.

Table 6 lists all the single-step selection experiments which were undertaken.

In studies of drug resistance in bacteria, yeast and free-living protozoa, calculations of the frequency

with which resistance to a particular drug arises have been carried out. To determine whether it is valuable to calculate such parameters for blood-form malaria parasites, the results of experiments in this study which employed 50 mg/kgm drug dose have been retabulated to compare the numbers of resistant lines to emerge in relation to the following: (i) the number of parasites originally inoculated into the mice (Table 7), and (ii) the mean number of parasites present per mouse at the time of the first drug injection (Table 8). In Tables 7 and 8 the selection experiments have been grouped according to the treatment of the parasite material, namely, untreated, cloned or U.V. irradiated.

The characteristics of the selection experiments yielding resistant lines

Of the four parasite strains used in this study, three developed resistance to pyrimethamine. These were strains 17X, 33X and N67.

Six separate pyrimethamine-resistant lines were obtained. Two of these resistant lines, 17X(Pr1) and N67(Pr2) were derived from untreated parasite material, while another two, 17X(Pr5) and N67(Pr6) were obtained from cloned parasite material. The remaining two lines, 33X(Pr3) and 33X(Pr4) were derived from U.V. irradiated parasite material.

Table 7 The appearance of pyrimethamine-resistant lines in relation to the number of parasites injected into each mouse.

[the figures in brackets are the number of resistant lines which were obtained]

Treatment of parasite material	Exp. No.	Sensitive parasite strain	The number of mice inoculated with 10^6, 10^7 or 10^8 parasites		
			10^6	10^7	10^8
Untreated	10	17X		25(1)	
"	11	"	30(0)		
"	18	33X		18(0)	
TOTAL			30(0)	43(1)	
Cloned	6	N67		30(1)	
"	7	"		16(0)	
"	13	17X		50(0)	
"	14	"		19(1)	
"	15	"		57(0)	
"	21	33X		50(0)	
TOTAL				222(2)	
UVirradiated	12	17X	30(0)		
"	19	33X		17(0)	
"	20	"			30(2)
TOTAL			30(0)	17(0)	30(2)

Table 8 The appearance of pyrimethamine-resistant lines in relation to the mean number of parasites present per mouse at the time of the first drug injection.

[The figures in brackets are the number of resistant lines which were obtained]

Treatment of parasite material	Exp. No.	Sensitive parasite strain	The number of mice with 10^7, 10^8 or 10^9 parasites present at the time of the 1st drug injection			Total No. of parasites of each type of parasite material subjected to drug treatment
			10^7	10^8	10^9	
Untreated	10	17X			25(1)	
"	11	"	30(0)			
"	18	33X			18(0)	
TOTAL			30(0)		43(1)	43.3×10^9(1)
Cloned	6	N67			30(1)	
"	7	"			16(0)	
"	13	17X		50(0)		
"	14	"			19(1)	
"	15	"		57(0)		
"	21	33X			50(0)	
TOTAL				107(0)	115(2)	125.7×10^9(2)
UV irradiated	12	17X	30(0)			
"	19	33X			17(0)	
"	20	"			30(2)	
TOTAL			30(0)		47(2)	47.3×10^9(2)

The frequency of development of pyrimethamine-resistance

Tables 7 and 8 both contain all the experiments carried out with a selection pressure of 50 mg/kgm of pyrimethamine. Table 7 shows that there is some degree of relationship between the number of parasites inoculated into each mouse and the number of emerging pyrimethamine-resistant lines. However, a stronger correlation can be seen in Table 8, between the size of the parasite population at the time of the first drug injection and the number of emerging resistant lines.

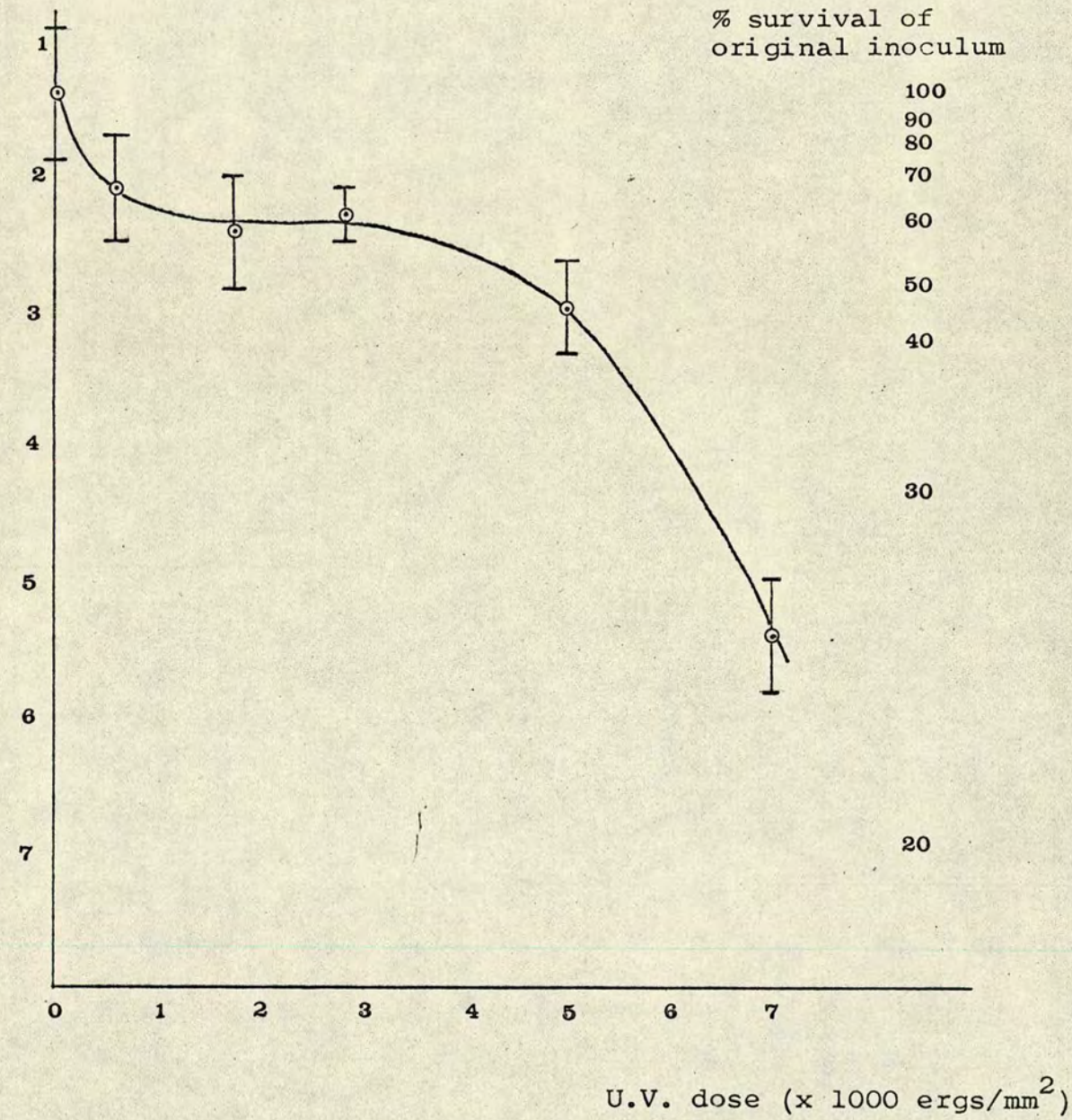
In Table 8 the number of parasites, of the four malarial strains studied, which were present at the time of the first drug injection have been summated. This summation gives the following as the frequency of development of resistance: one resistant line per 4.3×10^{10} untreated parasites subjected to drug treatment; and one resistant line per 6.3×10^{10} cloned parasites subjected to drug treatment, and one resistant line per 2.4×10^{10} U.V. irradiated parasites subjected to drug treatment.

Table 8 shows that a total of 6.8×10^{9} untreated parasites of strain 151BY were present at the time of the first drug injections, that is only one sixth of the number of parasites of the other 3 parasite strains studied which yielded one resistant line. The cause of the failure of 151BY to yield a resistant line will be discussed in a later section.

A U.V. dose-response curve was constructed to give background information on the effect of this treatment

Fig.4. U.V. dose-response Curve

Graph of mean time to reach 1% parasitaemia against U.V. dose. S.E. = standard error.
(Each result derived from 4 infections in T.O. mice).



on parasite viability (fig.4). Parasites of strain 33X were used for this study. From fig.4 it will be seen the large doses of U.V. would appear to affect parasite viability as measured with the modified Warhurst bio-assay, i.e. the calculation of the percentage of parasites surviving a given U.V. dose was obtained by calculating the ratio of the mean length of time between inoculation of parasites and the presence of a 1% parasitaemia for a group of mice receiving zero U.V. and of those mice receiving a particular level of U.V. treatment. Such a method of calculation assumes that the time taken by the infection to reach a 1% parasitaemia is directly proportional to the number of viable cells originally inoculated, and that the growth rate of parasites receiving zero or other doses of U.V. irradiations are the same.

Section II. The characterisation of pyrimethamine-resistant and sensitive lines

This section contains the results of the tests carried out on both pyrimethamine-resistant and sensitive lines to characterise their response to pyrimethamine and their GPI type. The results obtained with the simple tests devised to study the suspected variation among the parasite lines for some additional characters are also included. These additional characters were firstly the development site, secondly the effect of PABA on growth, and finally the response to sulphadiazine.

(a) Response to pyrimethamine

This study was undertaken to compare the response to pyrimethamine of the three drug-sensitive strains; 17X, 33X and N67, and the six drug-resistant lines obtained by selection.

The modified Warhurst bioassay was used in this study with an end-point parasitaemia of 1% (see p. 33). It will be recalled that the 'test period' is the interval between parasite inoculation and the recording of a 1% parasitaemia.

Tables 9a and 9b contain the results of the 37 tests carried out in nine experimental groups. Results are expressed as the length of the 'test period' observed, and the increase in the 'test period' after drug treatment. The results for individual mice employed in these tests are given in Table 23a, Appendix B.

Table 9a. Response to Pyrimethamine

A comparison of the duration of 'test periods' in infected mice receiving zero (controls), 2, 50, and 75 mg/kgms of pyrimethamine. Unless otherwise stated, all values are the mean result from 4 mice. Time in days. SE = standard error.

Parasite line	Experiment No.	Date of Experiment	Mouse Strain	'TEST PERIOD' ± SE Dose of pyrimethamine (mg/kgm) 0	2	50	75	Increase in 'TEST PERIOD' in comparison with controls. Dose of pyrimethamine (mg/kgm):- 2	50	75
17X	6	11.8.71	T.O.	2.4±0.49	4.8±0.28	8.4±0.24		2.4±0.57	6.0±0.55	
	7	18.10.71	T.O.	2.0±0.22	3.9±0.69	7.3±0.09		1.9±0.72	5.3±0.25	
	8	21.3.72	T.O.	2.2±0.22	4.2±0.53	8.0±0.23	9.7±0.38*	2.0±0.57	5.8±0.32	7.5±0.45
	9	17.8.72	T.O.	1.8±0.03	4.0±0.57	5.8±0.07	7.5±0.22	2.2±0.57	4.0±0.10	5.7±0.22
N67	2	1.6.71	C57	2.9±0.18	6.8±0.11			3.9±0.20		
	6	11.8.71	T.O.	2.3±0.09	5.7±0.25	7.5±0.36		3.4±0.27	5.2±0.37	
	7a	18.10.71	T.O.	2.1±0.12	5.3±0.58	6.7±0.35		3.2±0.60	4.6±0.37	
	7b	18.10.71	T.O.	1.7±0.11	3.7±0.33	5.6±0.55		2.0±0.35	3.9±0.58	
	8	21.3.72	T.O.	2.5±0.39	3.3±0.29	7.4±0.23	7.4±0.24	0.8±0.48	4.9±0.45	4.9±0.46
	9	17.8.72	T.O.	1.9±0.06	3.7±0.11	6.0±0.17	7.2±0.25	1.8±0.10	4.1±0.17	5.3±0.25
33X	5	28.7.71	C57	1.7±0.07	4.2±0.74	8.6±0.51		2.5±0.75	6.9±0.52	
	6	11.8.71	T.O.	2.5±0.33	5.3±0.24	8.1±0.21		2.8±0.41	5.6±0.40	
	7	18.10.71	T.O.	1.9±0.13	5.3±0.29	7.2±0.33		3.4±0.32	5.3±0.36	
	8	21.3.72	T.O.	2.9±0.35	4.8±0.70	7.9±0.13	9.0±0.51	1.9±0.78	5.0±0.37	6.1±0.62
	9	17.8.72	T.O.	2.3±0.18	5.0±0.39	9.5±1.00	10.7±0.47	2.7±0.42	7.2±0.20	8.4±0.50

Figures independently rounded

* Mean value of 3 mice

Table 9b. Response to Pyrimethamine

A comparison of the duration of 'test periods' in infected mice receiving zero (controls), 2, 50, and 75 mg/kgms of pyrimethamine. Unless otherwise stated, all values are the mean result from 4 mice. Time in days. SE = standard error.

Parasite line	Experiment No.	Date of Experiment	Mouse Strain	'TEST PERIOD' ± SE Dose of pyrimethamine (mg/kgm):- 0	2	50	75	Increase in 'TEST PERIOD' in comparison with controls. Dose of pyrimethamine (mg/kgm):- 2	50	75
17X(Pr1)	1	25.5.71	C57	2.5±0.48	2.9±0.35	6.7±0.33		0.4±0.59	4.2±0.58	
	3	16.6.71	C57	2.3±0.14	2.9±0.05			0.6±0.15		
	4	29.6.71	C57	1.9±0.06	2.2±0.16			0.3±0.15		
	6	11.8.71	T.O.	2.4±0.27	2.5±0.37	5.1±0.25*		0.1±0.47	2.7±0.37	
	7	18.10.71	T.O.	2.3±0.43	1.8±0.12	2.9±0.46		-0.5±0.45	0.6±0.62	
	8	21.3.72	T.O.	2.6±0.27	2.2±0.27	4.3±0.55	6.0±0.28	0.4±0.37	1.7±0.61	3.4±0.39
	9	17.8.72	T.O.	1.9±0.05	1.9±0.03	2.9±0.30	5.1±0.34	0.0±0.01	1.0±0.03	3.2±0.34
N67(Pr2)	3	16.6.71	C57	2.3±0.44	2.0±0.06			-0.3±0.45		
	4	29.6.71	C57	2.1±0.06	2.1±0.10			0.0±0.12		
	6	11.8.71	T.O.	2.5±0.17	2.3±0.09	3.0±0.12		-0.2±0.20	0.5±0.22	
	9	17.8.72	T.O.	2.3±0.09	2.8±0.28	2.5±0.03	3.1±0.16	0.5±0.30	0.2±0.11	0.8±0.20
33X(Pr3)	5	28.7.71	C57	2.7±0.43	3.3±0.41	6.2±0.16		0.6±0.60	3.5±0.46	
	6	11.8.71	T.O.	2.2±0.15	2.2±0.14	3.9±0.57		0.0±0.20	1.7±0.58	
	7	18.10.71	T.O.	1.5±0.05	1.6±0.03	1.8±0.05		0.1±0.06	0.3±0.07	
	8	21.3.72	T.O.	1.9±0.03	2.4±0.34	2.0±0.18*	2.6±0.40	0.5±0.35	0.1±0.18	0.7±0.40
	8	21.3.72	T.O.	2.3±0.07	2.2±0.13	3.2±0.26	3.4±0.39	-0.1±0.16	0.9±0.27	1.1±0.41
	9	17.8.72	T.O.	2.3±0.09	2.2±0.04	3.2±0.47	4.4±0.30*	-0.1±0.11	0.9±0.48	2.1±0.32

*Mean result from 3 mice

9b cont....

Table 9b cont.

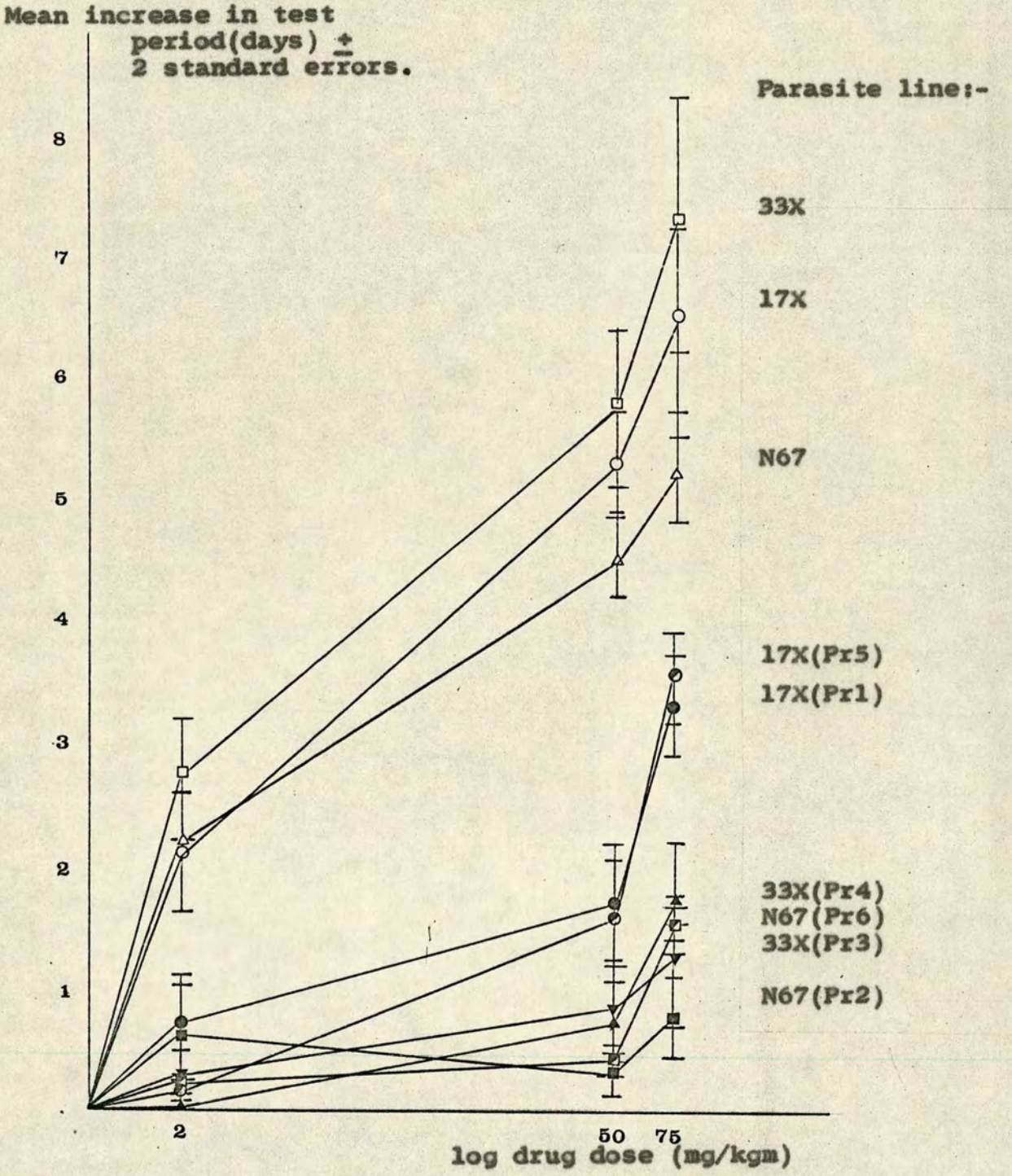
Parasite line	Experiment No.	Date of Experiment	Mouse Strain	'TEST PERIOD' ± SE Dose of pyrimethamine(mg/kgm);- 0	2	50	75	Increase in 'TEST PERIOD' in comparison with control. Dose of pyrimethamine (mg/kgm):- 2	50	75
33X(Pr4)	6	11.8.71	T.O.	2.2±0.09	2.2±0.06	3.3±0.24		0.0±0.12	1.1±0.27	
	9	17.8.72	T.O.	2.6±0.11	2.7±0.03	3.0±0.11	4.4±0.24	0.1±0.10	0.4±0.14	1.8±0.27
17X(Pr5)	9	17.8.72	T.O.	1.6±0.03	1.8±0.04	3.3±0.22	5.2±0.18	0.2±0.05	1.7±0.23	3.6±0.18
N67(Pr6)	9	17.8.72	T.O.	1.8±0.04	1.9±0.10	2.3±0.05	3.3±0.07	0.1±0.11	0.5±0.02	1.5±0.11

Figures independently rounded

Fig.5. Response to Pyrimethamine

Graph of mean results of modified Warhurst bioassays on 9 parasite lines. All experiments carried out in T.O.mice

A tabular presentation of these results is given in Table 23b, Appendix B.



Two strains of mice were used for these tests: C57 and T.O. mice. A summary of the 'test period' values obtained using T.O. mice is given in Fig.5. The methods of summation of the results from individual experiments and of calculating the accompanying standard errors is given in Appendix C.

Figure 5 indicates that the response of the pyrimethamine-resistant and sensitive parasite lines to pyrimethamine could be clearly distinguished using this test. However, it was found to be difficult to use this test to distinguish between levels of drug-resistance, as the order of increasing resistance of the parasite lines to the action of the drug varied with each drug dose given. In general, however, the results obtained with the 50 and 75 mg/kgm drug doses suggested that P.b. yoelii 17X and 33X were more sensitive to the action of pyrimethamine than P.b. nigeriensis N67, while the pyrimethamine-resistant lines derived from the 33X and N67 parasite material appeared to be more strongly resistant to pyrimethamine than the two lines obtained from the P.b.yoelii strain 17X.

(b) The Stability of resistance of the 6 pyrimethamine resistant lines

Since the development of the six pyrimethamine-resistant lines by selection, the lines have been maintained in the laboratory by blood and mosquito passage, with periods in liquid nitrogen. Some details are

given of the individual life histories of these lines.

17X(Pr1): This line was developed in June 1970. Initially the level of resistance of this line was followed with a 4 day suppressive drug test. During the period from June to September 1970, 17X(Pr1) underwent 3 mosquito and 36 rodent passages, and was kept for 6 rodent passages under a low level of drug pressure (5 mg/kgm administered on day 1 post-inoculum), without change in the response of the line to pyrimethamine. The level of resistance of this line was subsequently followed with a modified Warhurst bioassay. Between Octobers 1970 and 1972, this line underwent 55 rodent passages; 18 mosquito transmissions; and a 5 month period in liquid nitrogen without any significant reduction in its resistance to pyrimethamine.

N67(Pr2): This line was produced in June 1970. It has undergone 9 cyclical and 68 blood passages without changing its degree of resistance to pyrimethamine. Thirteen clones of this line were also tested for resistance to pyrimethamine and all proved to be as resistant to the drug as the original, uncloned line.

33X(Pr3): Line 33X(Pr3) was produced in June 1971. It has undergone 50 rodent passages, 5 mosquito passages, and two eight month periods in liquid nitrogen store without loss of resistance. A line cloned by dilution has been shown to possess the same level of pyrimethamine-resistance

as the original uncloned line.

33X(Pr4): This line was developed in June 1971, since then it has undergone 26 rodent and 3 mosquito passages without loss of resistance.

17X(Pr5): This line was isolated in November 1971. Three attempts to transmit the line through mosquitoes were unsuccessful. This lack of success can probably be explained by the fact that the line had undergone 15 blood passages since the previous mosquito transmission. The drug resistance of this line has remained stable through 15 blood passages.

N67(Pr6): Line N67(Pr6) was produced in November 1971. This line has undergone 2 mosquito and 15 rodent passages without change in its level of resistance to pyrimethamine.

(c) Enzyme type

Carter (1970) demonstrated that strain 17X could be distinguished from 33X and N67 by starch gel electrophoresis of the enzyme glucose-phosphate isomerase (GPI). The six pyrimethamine-resistant lines obtained in this study were found to possess the same GPI type as the parent strain from which they were derived (Table 14).

(d) Development site

The parasite lines were classified as either; R, developing mainly in reticulocytes; or M & R, developing in both mature and immature erythrocytes. The appearance

of blood infections from these types of development can be seen in Figs 6a and 6b.

A survey carried out in four T.O. mice per parasite line in August 1972, showed the development site of the parasite lines to be as follows:-

R: 17X, 33X, 17X(Pr1) and 33X(Pr4).

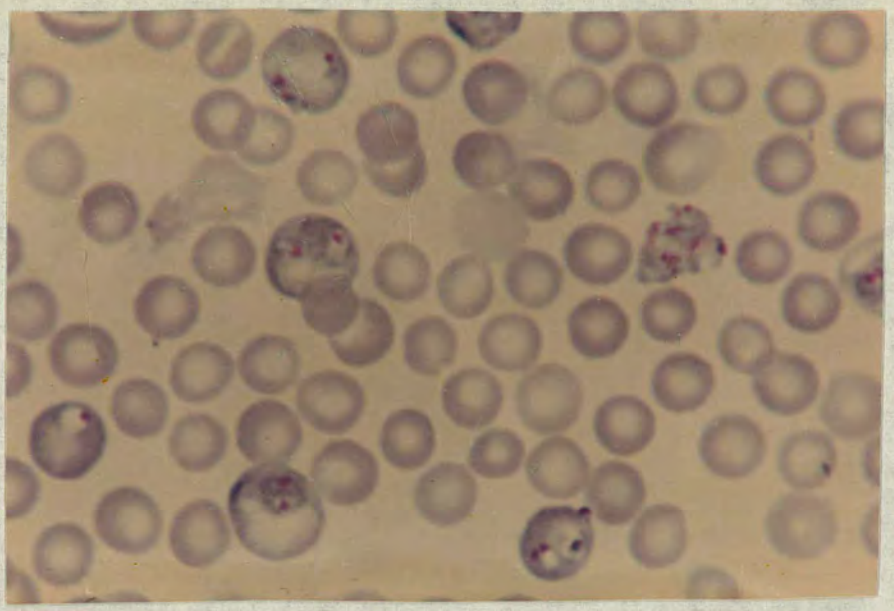
M & R: N67, N67(Pr2), 33X(Pr3), 17X(Pr5) and N67(Pr6).

On other occasions, the development site associated with a given line was usually found to be the same as that listed above, however occasionally a line normally of the R type was found to be extensively inhabiting mature erythrocytes. This change in development site was associated with parasite material which had undergone more than 12 blood passages. 17X(Pr5), is a line which had changed its development site type: when this line was produced in 1971 it was an R type.

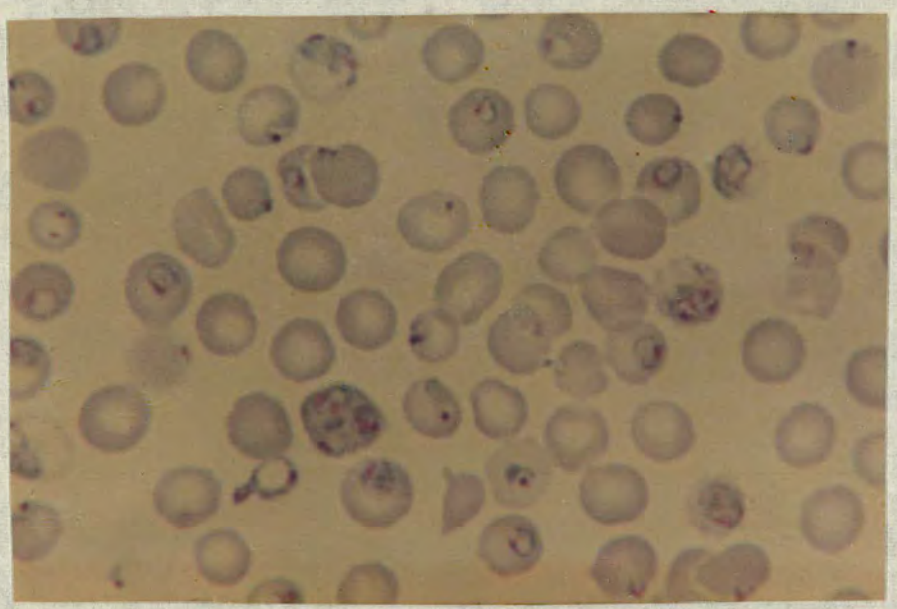
(e) The effect of PABA on growth

A comparison was made of the growth of each parasite line in two groups of "clean" mice, one group being maintained on a diet supplemented with a 0.05% solution of PABA in the drinking water (the standard mouse diet used in the laboratory), while the other group did not receive a PABA supplement at any time prior to the parasite inoculations or during the course of the infections. The index chosen to examine the effect of PABA was the ratio:

a.



b.



c.

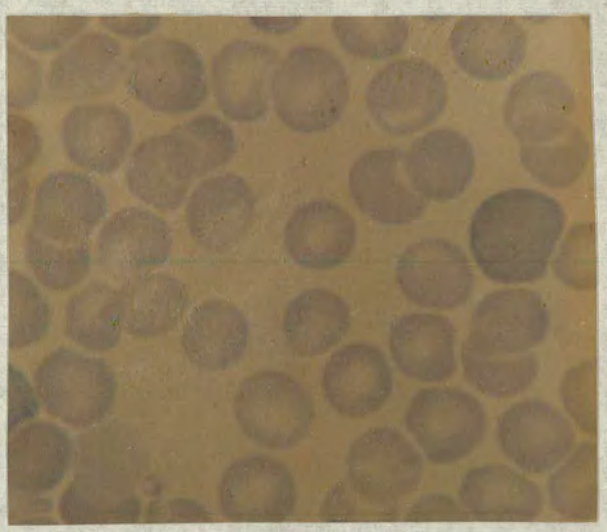


Fig.6. The development site characteristics of P.b. yoelii lines.

Blood pictures on day 4 post-inoculum of infections initiated with an inoculum size of 10^6 parasites.

Fig. 6a. R development site, that is development restricted to reticulocytes, of line 33X.
The parasitaemia in this type of infection follows the reticulocyte numbers in the blood, therefore, the rise of parasitaemia is slow and is dependent upon the reticulocyte response of the host to red blood cell destruction. This type of infection is not lethal.

Fig. 6b. M&R development site, that is development in both mature and immature erythrocytes, of line 33X-(Pr3). This infection type mainly inhabits reticulocytes when blood infection levels are low, but soon after becoming patent the parasites also invade mature erythrocytes, and thereafter parasitaemias rise steadily until 80% or more of the red blood cells are infected and the mouse host is severely anaemic.

Fig. 6c. Uninfected blood. Reticulocytes may be identified by their large size and strong blue staining with Giemsa's stain.

$$\frac{\text{Mean parasitaemia day 4 post-inoculum without PABA}}{\text{Mean parasitaemia day 4 post-inoculum with a 0.05\% PABA supplement}} \times 100$$

Table 10 lists the values of this ratio obtained in one experiment in T.O. mice. It will be realised that a ratio of the order of 100% would indicate that the line under study grew equally well up to day 4 post-inoculum in mice not receiving the PABA supplement as it did in the mice receiving the PABA supplement i.e. the parasite line was not dependent on the PABA supplement for its rate of growth, whereas a ratio very close to zero would indicate that the parasite line grew very poorly in mice not receiving PABA as compared with its performance in mice receiving the PABA supplement and therefore suggests that this parasite line is heavily dependent on the PABA supplement for its growth.

A comparison of the values of this ratio obtained in one experiment which tested 9 parasite lines on one occasion (Table 10) reveals that all 9 parasite lines tested experienced a slower rate of growth in mice not receiving the PABA supplement, compared with their growth rate in mice receiving PABA. However, their rates of growth were not equally reduced by the absence of the PABA supplement, as is demonstrated by the ratios listed in Table 10. The mean parasitaemia on day 4 post-inoculum of line 33X(Pr3) was only about 50% in the mice not receiving the PABA supplement of the mean parasitaemia in mice receiving the PABA supplement, whereas

Table 10. The Effect of PABA on the growth of 9 parasite lines: A comparison of the parasite growth (as estimated by mean parasitaemia day 4 post-inoculum) in 2 groups of mice, 1 group being maintained with a PABA Supplement in their drinking water.

Parasite line	Addition to mouse drinking water	Mean % parasitaemia of 4 T.O. mice on day 4 ± SE	Ratio*
17X	PABA	4.5 ± 0.61	11.1%
	NONE	0.5 ± 0.16	
17X(Pr1)	PABA	5.7 ± 0.96	0.0%
	NONE	0.0 ± 0.00	
17X(Pr5)	PABA	9.6 ± 2.34	9.4%
	NONE	0.9 ± 0.65	
N67	PABA	44.1 ± 8.48	4.5%
	NONE	2.0 ± 1.84	
N67(Pr2)	PABA	36.5 ± 2.40	10.1%
	NONE	3.7 ± 2.05	
N67(Pr6)	PABA	54.0 ± 4.67	2.8%
	NONE	1.5 ± 1.32	
33X	PABA	5.5 ± 1.10	9.1%
	NONE	0.5 ± 0.52	
33X(Pr3)	PABA	23.3 ± 2.69	57.9%
	NONE	13.5 ± 4.07	
33X(Pr4)	PABA	9.5 ± 1.34	2.1%
	NONE	0.2 ± 0.45	

$$\text{*Ratio} = \frac{\text{Mean parasitaemia day 4 post-inoculum without PABA} \times 100}{\text{Mean parasitaemia day 4 post-inoculum with a 0.05\% PABA supplement}}$$

the other parasite lines tested all experienced mean parasitaemias in mice not receiving PABA of between 0% and 11% of the mean parasitaemia in mice receiving the supplement.

On no other occasion were all the parasite lines tested simultaneously. From these results it was considered that line 33X(Pr3) might be in some way less dependent on a PABA supplement for its growth.

It was decided to examine a series of clones of this line for their dependence on PABA for growth, with a view to studying the genetics of this character. These results are included in a later section (page 78).

It was concluded that the capacity of line 33X(Pr3) for growth without PABA supplementation could not be directly related to the ability of the line to develop in mature erythrocytes, for lines N67, N67(Pr2) and 17X(Pr5) which all develop in mature erythrocytes, were found to have very limited growth in mice not receiving the PABA supplement.

When the effect of PABA on the growth of a resistant line was compared with its effect on the parasite strain from which it was derived, only 17X(Pr1) was found to require more PABA for growth than its parental line, 17X. 33X(Pr3) required less PABA for growth than 33X. The remaining resistant lines did not differ significantly in their requirements for PABA from the lines from which they were derived.

From these results 33X(Pr3) was classified as of low dependence on PABA for growth, while the remaining

8 lines which had been studied were classified as highly dependent on PABA for growth.

(f) The response to Sulphadiazine

Sulphadiazine is antagonised by PABA. When plasmodia blood infections in mice maintained on a 0.05% PABA drinking-water supplement were treated with high doses of sulphadiazine (200 mg/kgm) on day 0 post-inoculum, the subsequent parasite growth was the same as control parasite infections which had not received the drug treatment. It was therefore decided to reduce the PABA supplement to a tenth of the usual level i.e. a 0.005% solution.

Initial studies suggested that this lower level of PABA supplement allowed the same rate of parasite growth to a 1% parasitaemia end point as the usual 0.05% level. A comparison of the time taken by parasite infections to reach a 1% parasitaemia in C57 mice whose diet was supplemented with either a 0.05% or a 0.005% solution of PABA has been carried out using Students 't' test (see Appendix C). The results for this exercise being the growth rates of the "control" groups of mice in the modified Warhurst drug tests which did not receive treatment with pyrimethamine or sulphadiazine (see tables 23a and 25), in which all infections were initiated with an estimated 10^6 parasites.

Two "t" tests were carried out. In the first, the two samples to be compared were compiled solely on the

basis of the PABA supplement which they received, and both samples contained infections of a number of different parasite lines. The means of these two samples in reaching a 1% parasitaemia were 2.4 days (for the 0.05% supplement sample, sample size 32) and 3.0 days (for the 0.005% PABA supplement sample, sample size 72). The t value obtained for this test suggests that the difference between the sample means was too large to be due to chance alone (p = 1%).

In the second test, only the results of infections with strain 17X(Pr1) were assigned to the two samples, however the two sample sizes were smaller, namely 12 mice per sample, than in the first test. The t value for this test suggested that the difference of 0.9 days observed between the two sample means (0.05% PABA supplement sample mean was 2.3 days, while the 0.005% PABA supplement sample mean was 3.2 days) was not significant (p = 10%) with the smaller sample sizes.

Tables 11, 12 and 25 (Appendix B) reveal the extent of the variation in results obtained with the sulphadiazine drug tests. The summation of the results from individual experiments on parasite lines has been included in tabular form (table 12) as the size of the standard errors attached to the mean results does not facilitate their graphical presentation, as was possible with the results of the pyrimethamine drug tests.

An addition tabulation included on the results of the sulphadiazine drug tests ranks the results for

Table 11. Response to Sulphadiazine

A comparison of the duration of 'test periods' in infected mice receiving zero(controls), 25, 125 and 500 mg/kgm of sulphadiazine. All values are the mean results of 4 C57 mice unless stated otherwise. Time in days. SE=standard error.

Parasite line	Experiment No	Date of Experiment	Mouse Strain	'TEST PERIOD' ± SE Dose of sulphadiazine(mg/kgm):- 0	25	125	500	Increase in 'TEST PERIOD' ± SE in comparison with controls. Dose of sulphadiazine (mg/kgm):- 25	125	500
17X	2	21.3.72	C57	2.4±0.34	2.5±0.23	3.9±0.15	3.1±0.36	0.2±0.42	1.5±0.37	0.7±0.50
	3	15.5.72	C57	3.8±0.29	6.1±0.19	7.8±0.16	9.8±0.58	2.2±0.35	4.0±0.33	5.9±0.65
	6	17.8.72	C57	2.2±0.22	6.1±0.23	9.8±0.14	11.3±0.51	3.9±0.32	7.8±0.27	9.1±0.56
N67	3	15.5.72	C57	2.8±0.26	4.5±0.53	4.4±0.13	8.4±1.46	1.7±0.59	1.6±0.30	5.5±1.48
	4	3.8.72	C57	3.0±0.31	6.1±0.91	10.8±0.45	12.0±0.33	3.1±0.96	7.7±0.54	9.0±0.45
33X	2	21.3.72	C57	2.6±0.22	3.2±0.31	6.7±0.58	8.6±0.59	0.6±0.39	4.1±0.62	6.0±0.63
	5	9.8.72	C57	2.6±0.36	3.7±0.39	5.3±0.27	6.1±0.55	1.1±0.53	2.8±0.45	3.5±0.66
17X(Pr1)	1	23.2.72	C57	3.3±0.10	4.1±0.21	7.4±0.34	8.2±0.27	0.9±0.22	4.1±0.36	5.0±0.28
	2	21.3.72	C57	3.5±0.25	3.8±0.15	5.4±0.29	5.2±0.25*	0.3±0.28	2.0±0.39	1.8±0.35
	6	17.8.72	C57	2.8±0.02	5.3±0.25	9.4±0.22		2.5±0.25	6.6±0.22	
N67(Pr2)	3	15.5.72	C57	2.6±0.13	4.2±0.17	4.5±0.18	6.0±0.57	1.6±0.22	2.0±0.22	3.5±0.59
	4	3.8.72	C57	3.8±0.06	4.4±0.24	11.5±0.65	11.7±0.28	0.7±0.25	7.7±0.65	7.9±0.29
33X(Pr3)	1	23.2.72	C57	2.4±0.12	2.9±0.12	3.2±0.09	3.4±0.04	0.6±0.17	0.8±0.14	1.0±0.11
	2	21.3.72	C57	1.9±0.05	2.1±0.10	2.5±0.45	2.6±0.16	0.2±0.11	0.7±0.45	0.7±0.15
	4	3.8.72	C57	3.2±0.08	4.3±0.16	4.6±0.35	5.6±0.37	1.1±0.17	1.4±0.36	2.4±0.39
33X(Pr4)	5	9.8.72	C57	2.6±0.13	4.8±0.20	6.3±0.28	6.4±0.26	2.3±0.25	3.7±0.32	3.9±0.30
17X(Pr5)	6	17.8.72	C57	2.4±0.26	5.4±0.27	6.8±0.09	8.0±0.32 *	3.1±0.39	4.5±0.28	5.6±0.41
N67(Pr6)	3	15.5.72	C57	2.9±0.19	4.1±0.09	4.4±0.21	6.1±0.54	1.1±0.22	1.4±0.28	3.2±0.57
	4	3.8.72	C57	3.2±0.58	5.4±0.61	4.9±0.64	6.8±0.38	2.3±0.84	1.8±0.85	3.7±0.69

Figures individually rounded

* mean results from 3 mice

Table 12. Response to Sulphadiazine

Summation of results from individual drug tests for each parasite line. (For method of calculating mean increase in 'test period' and standard errors, see Appendix C). SE = standard error. Time in days.

Parasite line	No. of experiments carried out on parasite line	Dpse of Sulphadiazine given (mg/kgm):- 25		125		500	
		Mean increase in Test Period ± SE	limits of ± 2 SE	Mean increase in Test Period ± SE	limits of ± 2 SE	Mean increase in Test Period ± SE	limits of ± 2 SE
17X	3	2.3 ± 0.46	1.40 - 3.24	4.4 ± 0.78	2.86 - 5.98	5.2 ± 1.08	3.08 - 7.40
N67	2	2.4 ± 0.56	1.27 - 3.51	4.6 ± 1.19	2.26 - 7.02	7.2 ± 0.95	5.34 - 9.14
33X	2	0.8 ± 0.25	0.34 - 1.34	3.4 ± 0.39	2.66 - 4.22	4.7 ± 0.60	3.52 - 5.92
17X(Pr1)	3	1.2 ± 0.30	0.63 - 1.83	4.2 ± 0.59	3.04 - 5.40	3.6 ± 0.67	2.24 - 4.92
N67(Pr2)	2	1.3 ± 0.18	0.95 - 1.67	4.8 ± 1.13	2.58 - 7.10	5.7 ± 0.90	3.89 - 7.49
33X(Pr3)	3	0.7 ± 0.13	0.41 - 0.93	1.0 ± 0.20	0.56 - 1.36	1.4 ± 0.26	0.85 - 1.89
33X(Pr4)	1	2.3 ± 0.20	1.85 - 2.65	3.7 ± 0.28	3.17 - 4.29	3.9 ± 0.26	3.33 - 4.37
17X(Pr5)	1	3.1 ± 0.27	2.51 - 3.59	4.5 ± 0.09	4.27 - 4.63	5.6 ± 0.32	4.95 - 6.26
N67(Pr6)	2	1.7 ± 0.35	0.99 - 2.39	1.8 ± 0.25	1.32 - 2.32	3.4 ± 0.32	2.78 - 4.06

Mean figures individually rounded. For the results from individual mice see Table 25, Appendix B.

each parasite line in order of apparent increased resistance to sulphadiazine (table 13). This tabulation was carried out to determine whether a relationship existed between the drug test results obtained and some unknown factor varying consistently with time. Table 13 does suggest that the parasite lines did appear to be associated with increasing drug resistance (or conversely decreasing drug activity) over the period from February to August 1972 during which the tests were conducted.

From table 12 it will be seen that line 33X(Pr3) was the least affected by the sulphadiazine drug treatment, in that drug doses of 125 and 500 mg/kgm with infections of this line resulted in much smaller delays in reaching the 1% endpoint than with the other parasite lines studied.

In conclusion, therefore, despite the large variation in drug test results obtained, it was considered that line 33X(Pr3) was more resistant to sulphadiazine than the remaining 8 parasite lines studied. In a later section a discussion will be included of other factors which might have affected the results obtained with this drug, and caused the variation in results observed in repeat experiments on the same parasite line.

Table 13. Ranking of results obtained from individual experiments on each parasite line in order of increasing resistance to sulphadiazine indicated by these results.

Parasite Line	Experiments listed in order of increasing resistance to sulphadiazine as measured by the mean results in each experiment.(1)
17X	2 3 6
N67	3 4
33X	5 2(2)
17X(Pr1)	2 1 6
N67(Pr2)	3 4
33X(Pr3)	2 1 4
33X(Pr4)	5
17X(Pr5)	6
N67(Pr6)	3 4

Footnotes

1. Experiments carried out between February and August 1972. The dates of individual experiments were as follows:-

1	23.2.72
2	21.3.72
3	15.5.72
4	3.8.72
5	9.8.72
6	17.8.72

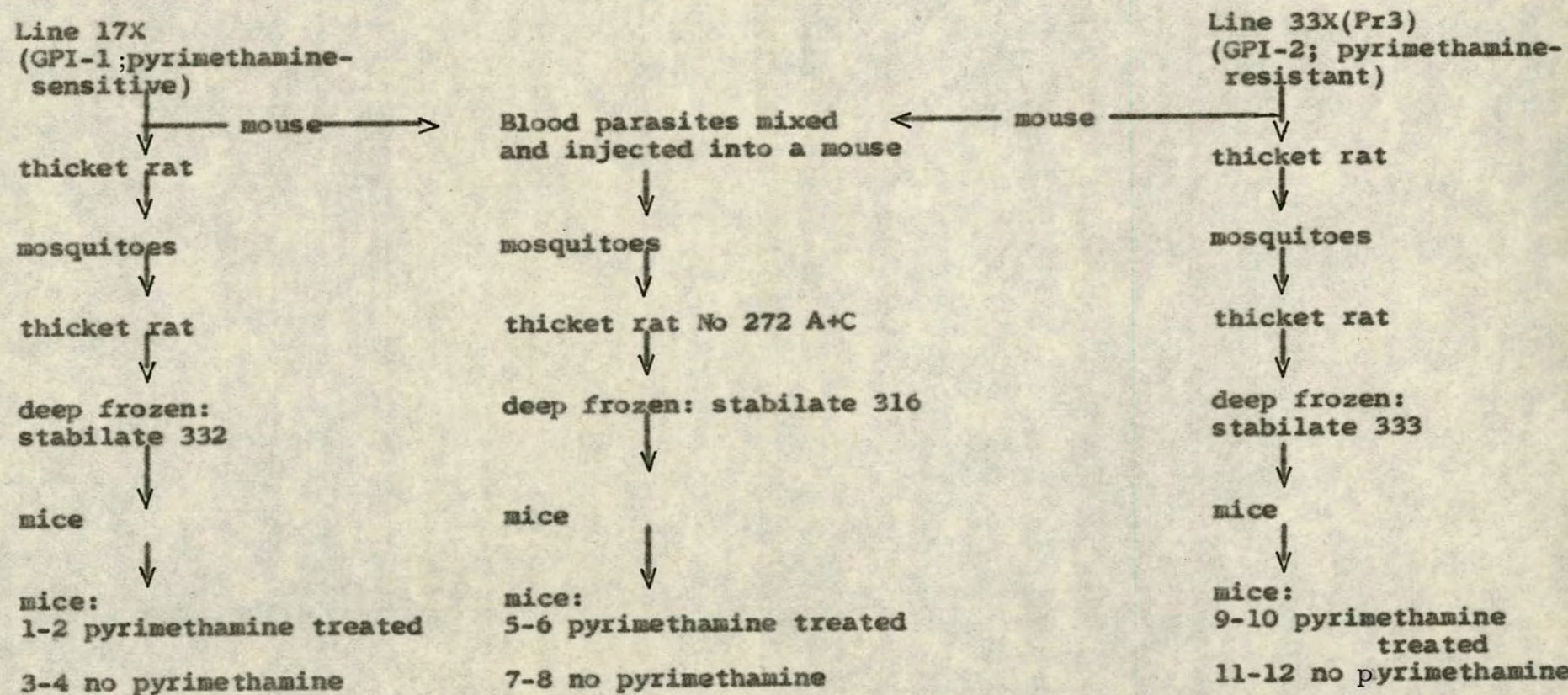
2. This ranking found for only 2 of the 3 drug doses, namely 25 and 125 mg/kgm, at 500 mg/kgm experiment 2 suggested line 33X to be more resistant to sulphadiazine than the result obtained in Experiment 5.

Table 14. Summary of characteristics of the 9 parasite lines used in this study

Parasite sub-species and line	GPI variant	Development site type	Degree of Dependence on PABA	Resistant to pyrimethamine	Resistant to sulphadiazine	Mosquito transmissible	Stable resistance to pyrimethamine	Treatment prior to selection for pyrimethamine resistance
P.berghei yoelli								
17X	1	R	high	NO	NO	YES	-	-
17X(Pr1)	1	R	high	YES	NO	YES	YES	NONE CLONED
17X(Pr5)	1	M&R	high	YES	NO	NO	YES	-
33X	2	R	high	NO	NO	YES	-	-
33X(Pr3)	2	M&R	low	YES	YES	YES	YES	UV.IRRAD.
33X(Pr4)	2	R	high	YES	NO	YES	YES	UV.IRRAD.
P.berghei nigeriensis								
N67	2	M&R	high	NO	NO	YES	-	-
N67(Pr2)	2	M&R	high	YES	NO	YES	YES	NONE CLONED
N67(Pr6)	2	M&R	high	YES	NO	YES	YES	CLONED

The results of these investigations to characterise the 9 parasite lines in this study are summarised in Table 14.

Fig.7. The procedure adopted for crossing line 17X with line 33X(Pr3)



SECTION III : GENETICS

1. Preconditions for successful crosses

A precondition for successful crosses in this study was found to be the use of two parasite lines with the ability to induce very heavy mosquito infections. This ability is enhanced by repeated mosquito transmission and declines immediately after cloning. As genetically uniform material is required for crossing experiments, the newly cloned parasite lines should undergo 3 or 4 successive mosquito transmissions before a cross is set up.

2. Analysis of a cross between line 17X and line 33X(Pr3)

Line 17X is GPI-1 and pyrimethamine-sensitive. Line 33X(Pr3) is GPI-2 and pyrimethamine-resistant. In addition, 17X develops mainly in reticulocytes and has a strong dependency on PABA for growth, whereas 33X(Pr3) can develop in both mature and immature erythrocytes and is of low dependency on PABA for growth. Line 33X(Pr3) probably acquired its PABA dependency and development site characteristics at the same time as resistance to pyrimethamine.

The procedure followed for conducting the cross and recovering the products is given in Fig.7.

A GPI-type analysis of the blood infection of thicket rat 272 A+C showed equal proportions of GPI-1 and 2 parasites among the products of the cross.

The results of the test to indicate whether recombination of the parasite lines had occurred, are set out in Table 15. These suggested the presence of parasites with a recombinant combination of characters, namely, GPI-1 and pyrimethamine-resistant, among the products of the cross.

Table 15.Results of the simple suppressive tests carried out on parasites from Stabilates 332, 316 and 333

Infection derived from Stabilate No,	Parasite line	Mouse No.	Pyrimeth-amine treated	Parasites present day 4 post-inoculum	GPI-type of para-sites present on day 4 post -inoculum
332	17X	1-2	YES	NO	-
		3-4	NO	YES	1
333	33X(Pr3)	9-10	YES	YES	2
		11-12	NO	YES	2
316	Products of the cross	5-6	YES	YES	1&2
		7-8	NO	YES	1&2

Forty clones were derived from the products of the cross, these are termed the product clones, and 20 from each of the pure lines. All these clones were successively characterised for GPI-type, response to

pyrimethamine and development site. Twenty-seven of the product clones and all of the pure line clones were examined for their degree of dependency on PABA for growth.

The GPI-type and pyrimethamine-response characteristics of all the clones are recorded in Table 16.

Table 16: Characteristics of Clones for GPI-type and response to pyrimethamine

Sens = pyrimethamine-sensitive

Res = pyrimethamine-resistant

Origin of clone	No. of clones isolated	Classes of Parasite:-			
		GPI-1 Sens	GPI-1 Res	GPI-2 Sens	GPI-2 Res
17X	20	20			
33X(Pr3)	20				20
Products of the cross	40	0	14	3	23

The 40 product clones consisted of 23 clones with the parental combinations of these characters and 17 recombinant clones. However, no product clones of the 17X parental combination, that is GPI-1 and pyrimethamine-sensitive, were obtained. Many more GPI-1, pyrimethamine-resistant recombinant parasites were among the cloned products than the GPI-2, pyrimethamine-

sensitive class of recombinant parasites.

The clones derived from the two pure parasite lines were associated with the same development site as the lines from which they were derived. The development sites of the 40 product clones are listed in Table 17 in conjunction with their GPI type and pyrimethamine-response character.

Table 17: The Development Site of the Clones Studied

Res = pyrimethamine-resistant

Sens = pyrimethamine-sensitive

R = develops in reticulocytes

M&R = develops in mature and immature erythrocytes

V = variable development site, i.e. some infections R, others M&R.

Origin of clones	No of clones	No of clones of each combination of parental characters								Clones with non-parental characters
		GPI-1				GPI-2				GPI-1
		Sens		Res		Sens		Res		Res
		R	M&R	R	M&R	R	M&R	R	M&R	V
17X	20	20								
33X(Pr3)	20								20	
Cross Products	40			2	9	3		1	23	2

The development site character did not always segregate with the pyrimethamine-response character. However, as the product clones were predominantly R development site and pyrimethamine-sensitive, or M&R development site

and pyrimethamine-resistant, there is a suggestion of linkage between these two characters.

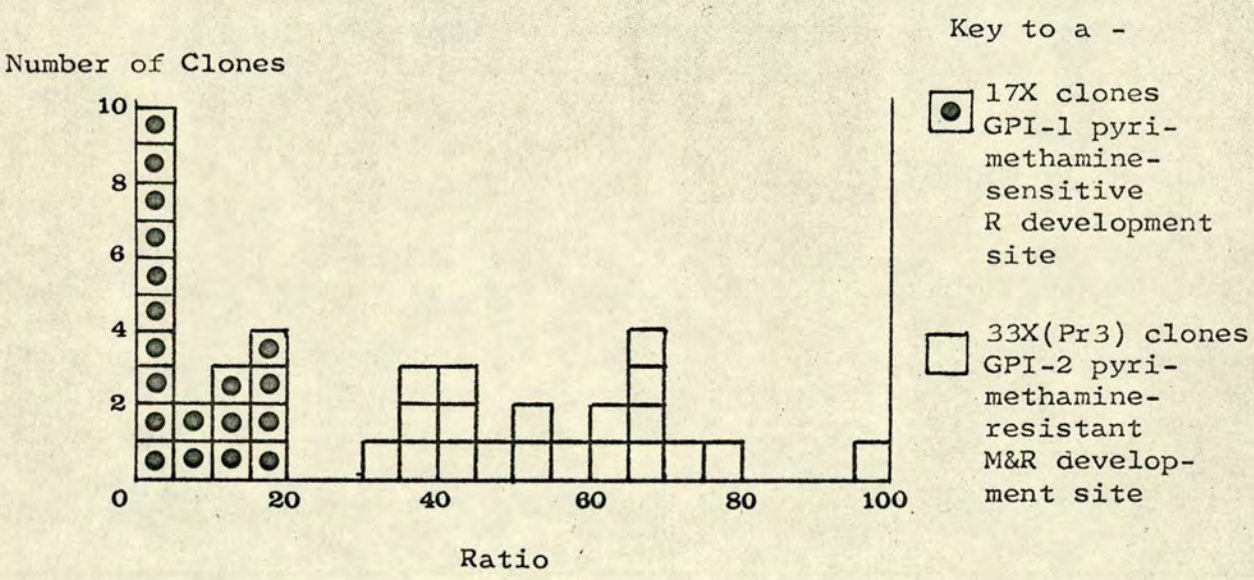
The effect of PABA on the growth of the pure line and product clones was examined. The method and ratio used were the same as those used previously to compare the effect of PABA on the growth rates of parasite lines and strains (see page 37). It will be recalled that for each parasite line a comparison was made of the growth of the parasite line in two groups of "clean" mice, one group being maintained on a diet supplemented with a 0.05% solution of PABA in the drinking water, while the other group did not receive a PABA supplement at any time prior to the parasite inoculations or during the course of the infections. The index used to compare the effect of PABA on the growth of each cloned parasite line was the following ratio:-

$$\frac{\text{mean parasitaemia day 4 post-inoculum without PABA}}{\text{mean parasitaemia day 4 post-inoculum with a 0.05\% PABA supplement}} \times 100$$

The results on the effect of PABA on the growth of parasite clones were all obtained in one experiment and proved to be complicated. An unexpectedly broad range of values for the index ratio was obtained for each set of clones (i.e. the 17X pure-line clones; 33X(Pr3) pure-line clones, and the product clones derived from the cross of these two pure lines). However, as can be seen from Fig.8, histogram a, the range of the ratios obtained for each set of pure line clones did

Fig.8. Histograms of the effect of PABA on the growth of pure line 17X and 33X(Pr3) clones, and on cloned products of a cross between these two parasite lines, cross tabulated with other characteristics of these clones.[1]

Histogram a: Pure line clones - the effect of PABA on the growth of pure line clones as measured by Ratio.[2]

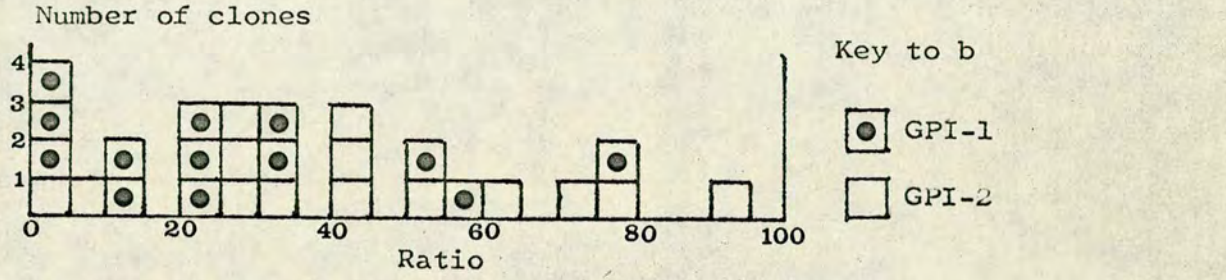


It will be seen from histogram a that the range of ratio values obtained for each set of clones did not overlap, for whereas the range of ratios for 17X clones extended from 0% to 20% the range for 33X(Pr3) clones was from 30% to 100%

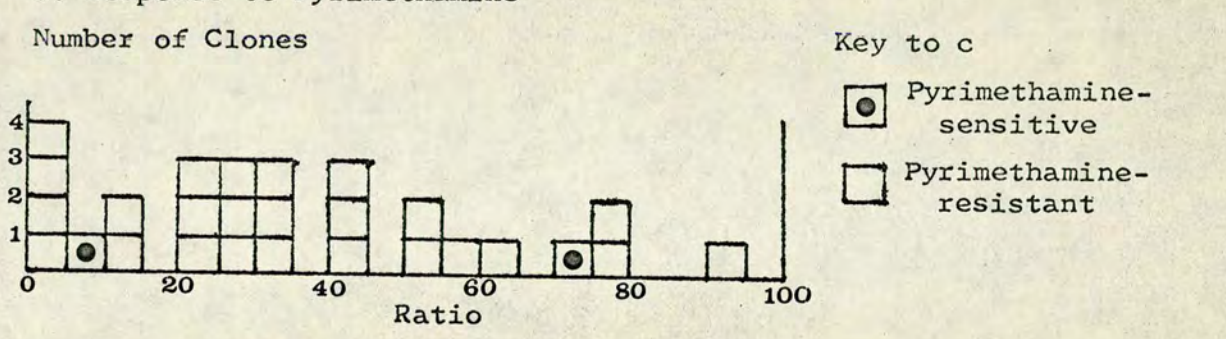
1. The full results of the experiments summarised in Fig.8 are included in Tables 27, 28, and 29 in Appendix B.
2. Ratio = $\frac{\text{Mean parasitaemia day 4 post-inoculum without PABA}}{\text{Mean parasitaemia day 4 post-inoculum with a 0.05\% PABA supplement}} \times 100$

Histograms b, c and d: Product Clones - the effect of PABA on the growth of clones derived from the products of a cross, between lines 17X and 33X(Pr3) as measured by the Ratio^2, cross tabulated by:-

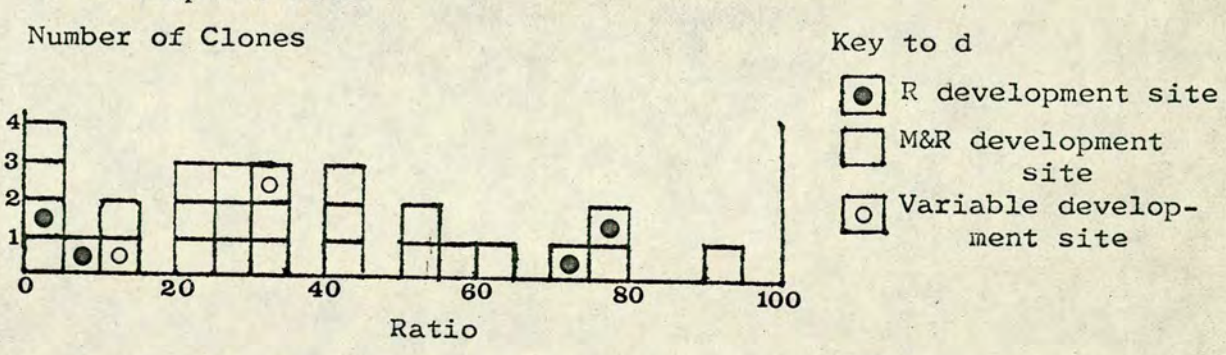
b. GPI-type



c. Response to Pyrimethamine



d. Development Site



Histograms b, c and d demonstrate the distribution of ratio values obtained for the product clones, i.e. parasite growth without PABA measured as a percentage of the growth on the standard PABA diet, separately cross tabulated by the 3 characteristics by which the parental lines differed. Each product clone is represented by a square in the same position in the 3 histograms. It will be observed that the 3 characteristics making up the "17X phenotype" are represented by ☐ whereas the "33X(Pr3) phenotype" is represented by ◉. Thus an examination of the shading pattern of histograms b, c and d allows the product clones which exhibited a non-parental combination of characteristics to be distinguished.

not overlap.

The distribution of the ratios obtained for the product clones (see Fig.8, histogram b) extended from 0% to 95%, that is the absence of a PABA supplement appeared to completely prevent the growth of some clones, to have some effect on the growth rates of other clones, and to have negligible effects on the growth of others.

These results of the effect of the PABA supplement on the growth of the product clones were examined for evidence of linked segregation of GPI-type, response to pyrimethamine and development site, (Fig.8, histograms b, c and d respectively), but no evidence for linked segregation of these characters was found. However, of the 40 product clones obtained, only 27 clones were examined with respect to the effect of PABA on their growth, and furthermore the majority of the clones not selected were of one class of parasite, namely GPI-2, pyrimethamine-resistant and M&R development site. Therefore special care must be taken in the interpretation of these results.

No estimates were made of the standard errors attached to the index measuring the dependence of the cloned lines on PABA as each mean index value was calculated from parasitaemias recorded in 2 groups each containing only 3 mice. The results obtained for all the individual mice involved in the experiments are however included in Tables 27 - 29 in Appendix B.

In summary, therefore, clones of both recombinant and parental combinations were obtained from the products of this cross, although one class of a parental combination of characters was not represented among the product clones. The characters believed to have been acquired by the 33X(Pr3) line at the same time as pyrimethamine-resistance did not always segregate with the pyrimethamine-resistance character. There was some evidence of linked segregation between the pyrimethamine response and development site characters. A discussion of the numbers and classes of parasites obtained among the product clones will be included in a later section.

3. Analysis of 7 clones derived from a cross between line 17X(Pr1) and line N67.

Line 17X(Pr1) is GPI-1 and pyrimethamine-resistant, whereas line N67 is GPI-2 and pyrimethamine-sensitive.

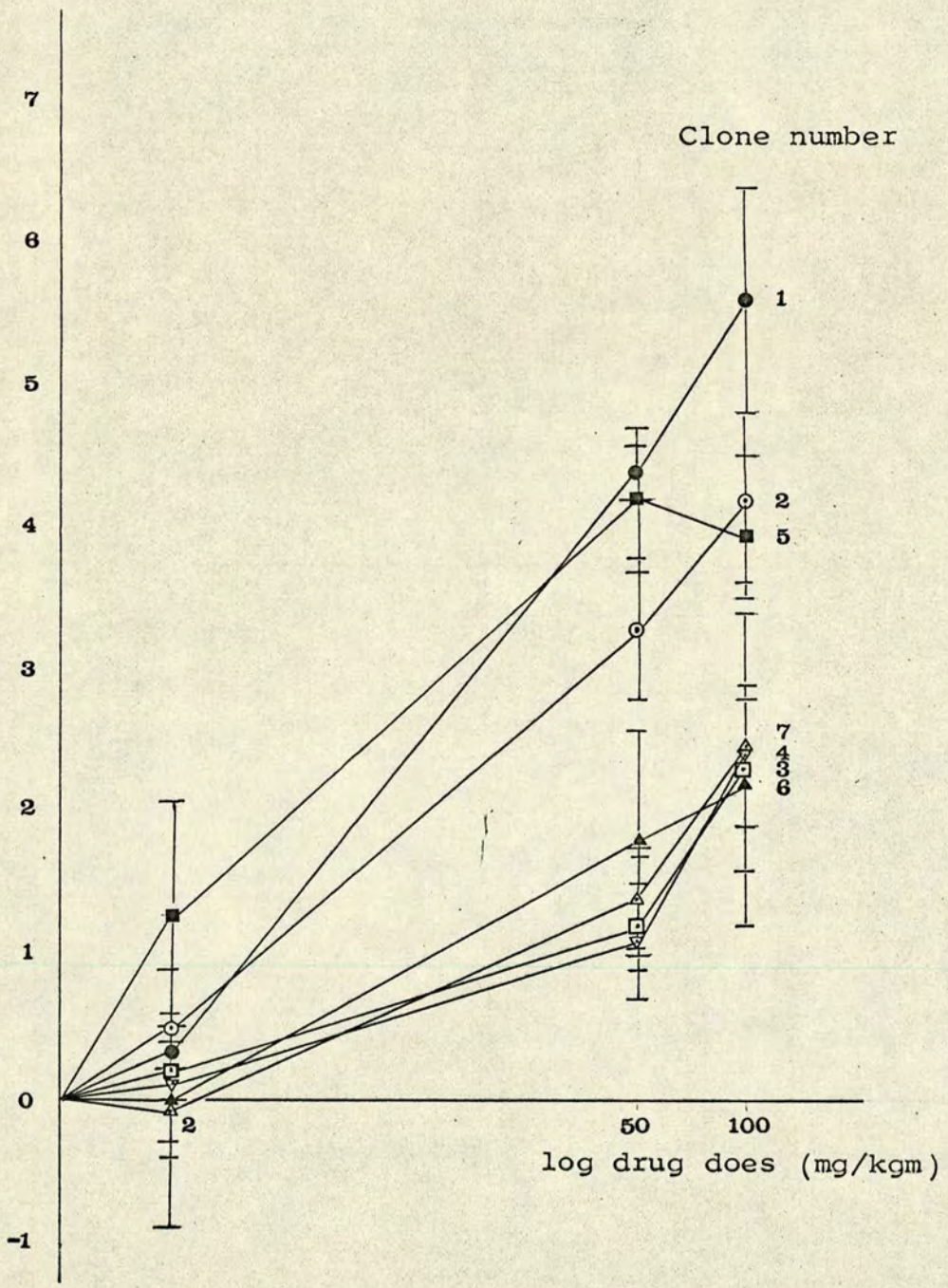
This cross was carried out by Mr A.Oxbrow. Details of the procedure for conducting the cross and recovering and cloning the products of the cross have been described elsewhere (Oxbrow, 1972).

To examine if more than one level of resistance or sensitivity to pyrimethamine was present among the cloned products of this cross, the response of 7 product clones to pyrimethamine was examined with the modified Warhurst bioassay.

Fig.9. Response to Pyrimethamine - Graph of the results of modified Warhurst bioassays on 7 clones derived from the products of a cross between lines 17X(Prl) and N67. (Each drug treatment was given to 4 mice infected with the same clone)

For the results of individual mice and a tabular presentation of the results in this figure, see Tables 26a and 26b, Appendix B.

Mean increase in the 'test period' ± 2 standard errors



The mean results obtained with this drug test are included in Table 18, and summarised in Fig.9. Individual mouse results obtained in these drug tests are included in Table 26, appendix B.

From Fig.9 it will be seen that the drug tests carried out on the 7 product clones only distinguished one pyrimethamine-resistant and one pyrimethamine-sensitive group of product lines.

Table 18. Response to Pyrimethamine of 7 clones derived from the products of a cross between lines 17X(Pr1) and N67.

A comparison of the duration of 'test periods' in infected mice receiving zero (controls), 2, 50 and 100 mg/kgm of pyrimethamine. Unless stated otherwise all values are the mean results of 4 mice. Time in days*

Parasite Clone No.	GPI type	Date of Experiment Parasite Inoculation	Mouse Strain	'TEST PERIOD' ± SE Dose of Pyrimethamine(mg/kgm):-				Increase in 'Test Period' in comparison with Controls, ± SE. Dose of Pyrimethamine (mg/kgm):-		
				0	2	50	100	2	50	100
1	1	2.3.72	T.O.	1.8±0.10	2.1±0.12	6.2±0.07	7.4±0.38	0.3±0.16	4.4±0.12	5.6±0.40
2	2	2.3.72	T.O.	2.5±0.24	3.0±0.33	5.8±0.02	6.7±0.15	0.5±0.41	3.3±0.24	4.2±0.28
3	1	2.3.72	T.O.	1.7±0.08	1.5±0.04	2.9±0.14	4.0±0.54	-0.2±0.09	1.2±0.16	2.3±0.55
4	1	2.3.72	T.O.	1.8±0.04	1.7±0.04	≠2.9±0.19	4.2±0.56	0.1±0.06	1.1±0.18	2.4±0.56
5	2	2.3.72	T.O.	1.9±0.14	3.2±0.35	6.1±0.18	5.9±0.20	1.3±0.38	4.2±0.23	4.0±0.25
6	1	2.3.72	T.O.	2.2±0.29	2.2±0.34	4.0±0.24	4.4±0.11	0.0±0.45	1.8±0.38	2.2±0.30
7	1	2.3.72	T.O.	1.8±0.13	1.7±0.06	3.2±0.11	4.3±0.23	-0.1±0.14	1.4±0.17	2.4±0.27

* For the complete experimental results see Appendix B, Table 26a.

≠ mean result of 3 mice.

DISCUSSION

Before proceeding to a discussion of the genetic studies, it is necessary to examine the results obtained with the selection experiments for pyrimethamine-resistance, and the characterisation studies.

(1) Selection Experiments

A large number of experimental malaria parasite lines have been developed which are resistant to one of the standard antifolate drugs used in malaria chemotherapy, using a selection technique (see Introduction for development of proguanil and pyrimethamine-resistance, while resistance to cycloguanil has been developed by Peters, 1965c; Thompson et al, 1965b; Bishop, 1966a and 1966b, and Richards, 1966b).

The most common selection method employed has been to subject a parasite line to gradually increasing drug pressure over a number of blood passages. For example, Bishop and Birkett (1947) obtained a 40 fold increase in resistance to proguanil in a line of P.gallinaceum by passaging the strain through chicks every 2 or 3 days with the largest dose of proguanil which was without effect upon the parasites when given over a long period (0.025 mgm/ 20 gm body weight). After one month the strain was found to be able to tolerate higher drug doses and therefore the drug dose was gradually increased, until after 4½ months parasites, which were normal in appearance, were

developing in chicks receiving two daily doses each of 1.0 mgm/20 gm body weight of chick, the largest dose the host could tolerate. On the other hand Williamson et al (1947), who obtained a proguanil-resistant line of P.gallinaceum at the same time as Bishop and Birkett, used a selection dose in one of their experiments in the region of the maximum tolerated by the host chicks, throughout the selection period. An alternative selection method is to apply selection pressure during only one blood passage, as Young (1957), Burgess and Young (1959) and Diggens (1970) have shown that resistance to pyrimethamine can emerge if a large number of parasites are treated with a single period of heavy drug treatment.

In this study the single session selection method was adopted, and altogether 616 infected mice were subjected to 4 doses of between 50 and 200 mg of pyrimethamine per kgm body weight, and six drug-resistant lines were obtained. These very heavy drug doses were used, as with this technique it is essential that no drug-sensitive parasites should "break through" the drug treatment. Furthermore, as it was intended to use the resistance as a genetic marker, it was hoped that this procedure would select lines exhibiting only a very high degree of resistance to pyrimethamine compared with the parent line.

The selection experiments carried out in this study were analysed to give a measure of the frequency with which pyrimethamine resistant lines emerged. Previously,

Rollo (1952a) had reported that resistance to pyrimethamine had developed more rapidly when the drug was given during heavy P.berghei infections, while Martin and Arnold (1968) established a clear relationship between the size of the parasite population at the time of the drug treatment and the development of resistance to a single dose of pyrimethamine during experiments with P.falciparum infections in non-immune volunteers.

After Coulston and Manwell (1941), Downs (1947), and possibly Demidowa (1934) had shown that individual blood parasites could be used to initiate blood infections, Bishop carried out investigations on the frequency of development of drug resistance using cloned parasite lines produced in this way. In 1958, Bishop reported on the development of resistance to metachloridine. By carrying out a comparison of the development of resistance in populations of normal parasites and of populations composed of mixtures of known numbers of resistant and normal parasites, Bishop suggested "that the pattern of development of this resistance in normal populations could be explained by selections of mutations of a frequency of less than 1 in 5×10^7 or probably less than 1 in 10^9 parasites."

Bishop (1962), extended this study to include the development of resistance to the antifolates, proguanil and pyrimethamine. The selection technique used involved exposing clone-derived material to a series of drug

treatments with intervening periods of growth in untreated hosts. Bishop reported that there was some evidence that the rate of development of resistance to proguanil depended upon the inoculum size used to passage the line, although a similar relationship was not found in the development of resistance to pyrimethamine. Bishop's experiments also showed that high doses of antifolates did not prevent the development of drug-resistance as had been suggested by Rollo(1952b) and Covell et al (1955).

In order to study the frequency of emergence of pyrimethamine-resistance estimates were made in the present study of the number of parasites inoculated into the host mice at the start of the selection experiments and of those present at the time of the first drug injection. A relationship was apparent between the number of parasites present at the start of the drug treatment and the emergence of the resistant lines. No difference in the frequency with which resistant lines emerged was detected among the parasite populations receiving the three selection procedures. However, the frequencies obtained were as follows: one resistant line per 4.3×10^{10} untreated parasites; one resistant line per 6.3×10^{10} parasites derived from cloned material, and one resistant line per 2.4×10^{10} parasites which had been subjected to ultraviolet irradiations. Thus a lower incidence of resistance was suggested by this study than that obtained by Bishop for metachloridine resistance.

It is possible however that the use of a single session of drug treatment for the selection technique may influence the frequency with which resistant lines are obtained. For example, this method would not allow highly resistant lines to arise through the gradual accumulation of heritable changes over a number of generations, and it might also allow certain random events to eliminate resistant cells, for example, the newly emerged resistant parasites might fail to invade a host erythrocyte or the resistant parasite might be destroyed by macrophagic or antibody attack. The 6 pyrimethamine-resistant lines obtained in this study were sub-inoculated from fleeting patent infections arising after the selection procedure, which suggests that the immune responses of the host were active at the time the resistant lines emerged, and that other resistant lines may have been destroyed before producing patent blood infections.

Of the four parasite strains employed in this study, only 151BY failed to yield a resistant line. However, fewer parasites of this strain were subjected to drug treatment and therefore this failure to obtain a resistant line could be due to an inherent inability of this strain to develop a resistance to pyrimethamine, or more likely, to too small a population of parasite cells being subjected to drug pressure.

Some measure of the frequency with which a drug-resistant line emerges after selection pressure is useful

in determining the origin of the resistance, because the use of selection agents to obtain specific variants inevitably obscures the origin of that variant. For a long time a controversy was maintained concerning the adaptation or mutational hypotheses of the origin of bacterial variation (Hayes, 1964). Originally, the adaptation hypothesis was the more widely held. This stated that each cell in a population had a finite chance of reacting to the presence of the selective agent in such a way as to become resistant to the action of that agent. Gradually, however, experimental results pointed to a mutational origin of variation. The mutational hypothesis states that variation arises as a result of random, spontaneous events unrelated to the environment of the organism and the variants that do arise are revealed by the selective agent removing all other types of organism present. There is now a considerable body of evidence showing that stable bacterial variation is exclusively due to spontaneous mutation (reviewed by Hayes, 1964). One of the most convincing experiments revealing the mutational origin of variation in bacterial studies was carried out by Lederberg & Lederberg (1952) using a replica plating method to isolate streptomycin and phage-resistant Escherichia coli variants without directly exposing the bacteria at any time to any selective agent, thus demonstrating the occurrence of spontaneous mutants.

This controversy is not however completely resolved. Hinshelwood (1946) put forward a further adaptationist hypothesis suggesting that contact of sensitive bacteria with a drug may shift the normal equilibrium of cellular chemical reactions to a new equilibrium which is less susceptible to the action of the drug. Furthermore, due to the distribution of the parent cytoplasm to both daughter cells during binary fission, this altered equilibrium could be perpetuated for a number of generations. No proof of this type of adaptation has as yet been found. However, there is no doubt that certain adaptive mechanisms do occur, but the resulting changes in the phenotype produced by the influence of a specific environment are not usually maintained after the transfer of the variant form to a different environment. Furthermore, such adaptive changes generally occur at a much higher frequency than mutational events.

The methods which have been used to expose the mutational basis of variation in bacterial studies are not at present available in malarial genetics. However, some information is available which strongly suggests that pyrimethamine-resistance can arise by mutation. Firstly, as mentioned previously, resistance to all of the antifolate antimalarial drugs have been shown to be stable through blood and mosquito passage, for although there have also been reports of pyrimethamine-resistant

lines which are labile (Rollo, 1955b, and Bray 1955), Peters (1970a) has suggested that this apparent lack of stability might be the result of a PABA deficiency in the diets of the mice used to maintain these lines. In the present study all six of the resistant lines obtained by selection were found to be stable during blood passage, and periods in the deep freeze, while the five lines which successfully underwent mosquito transmission also retained their resistance through that process. Secondly, the very low frequency with which resistance lines emerge suggests a mutational origin for these variants, as does the suddenness with which they are reported to appear (Young, 1957; Burgess and Young, 1959; Bishop, 1962, and Diggens, 1970). The ability of cloned parasite lines to yield pyrimethamine-resistance under selection pressure, which was first demonstrated by Bishop (1962) from cloned lines obtained in 1958, strongly suggests the mutational origin of the resistance. In the present study 2 pyrimethamine-resistant lines were obtained from cloned parasite material, one of the clones being obtained by a dilution method one month before the selection experiment commenced which suggests that the resistant line may have arisen during the course of the experiment.

An attempt was made in this study to employ mutagens to increase the frequency with which variant parasite types were obtained. Hawking (1966) attempted to use mustine to induce chloroquine-resistance in blood parasites

of P.berghei. He injected 5 or 10 mg/kgm of mustine into mice by the intraperitoneal route on the first and fifth days post-inoculum. Chloroquine was administered at a continuous low level in the mouse diet. After eight months involving 38 passages, there was no resistance and the line was discarded. However, no test was carried out to determine whether this chemical was mutagenically active to parasites within erythrocytes in the doses or route of injection by which it was administered.

Ultraviolet and X-irradiations have been used with malaria to produce inactivated sporozoite preparations to stimulate the immune response of the parasite's host (Mulligan et al, 1941; Russell and Mohan, 1942; Richards, 1966a, and Nussenzweig et al, 1967).

An attempt was made in this study to select an ultraviolet irradiation dose and administration method which could be shown to have some effect on parasite development. The preliminary investigation on the effect of U.V. irradiations on the viability of the erythrocytic parasites suggested that large doses of U.V. did kill blood-form parasites, although the shape of the dose-response curve was unusual. The dose-response curves of U.V. on organisms able to repair U.V. damage generally show a slow decline in viability at low doses of U.V. which becomes more rapid as U.V. dose increases. This response is interpreted as showing the initial efficiency of the repair system breaking down as the

it reached the parasite material.

When the effect of U.V. treatment on the frequency of development was investigated it was found that there appeared to be little difference in the frequency of development of pyrimethamine-resistance from U.V. treated and untreated parasites. The mutants obtained after drug-selection of U.V. treated parasite material could be a mixture of spontaneous and U.V. induced mutants. The number obtained would equal $x\,n + y\,m$ (1)

where: x and y are the frequencies of development of spontaneous and U.V. induced pyrimethamine-resistant mutants; n is the number of parasites subjected to drug pressure, and m, the number of parasites surviving the U.V. treatment. Due to the delay of 5-8 days between inoculation of the U.V. treated parasites and the administration of the first drug injection, there were 200 to 1,000 more parasites present at the time of the first drug injection than when the parasites were originally inoculated. If the small difference in the frequency of development of pyrimethamine-resistance from U.V. treated parasites and non-U.V. treated parasites which was obtained in this study is substituted into equation 1, a 36 fold increase in the frequency of development of resistance due to the U.V. treatment is found. This calculation is only included to illustrate how large an effect the U.V. treatment must have on the frequency of development of resistance before significant differences will be observed in this selection system, between the

numbers of resistant lines obtained from untreated and U.V. treated parasites.

The effect of U.V. treatment might be more easily demonstrated if the time period between inoculation of the parasites and the first administration of the drug, which is needed to allow the U.V. induced mutants to become manifest, was reduced to 3-4 days. It would also be possible to enlarge the number of parasites subjected to U.V. in a given experiment, but as it is felt that the size of the parasite inoculum should not exceed 10^8, so that a large proportion of the inoculated parasites contribute to the new blood infection, the selection experiment could most usefully be enlarged by injecting the parasites into 100-200 mice.

In the present study two resistant lines were obtained from parasites which had been exposed to U.V. irradiations. However, as so few resistant lines were obtained and as the incidence of resistance appeared very similar to that for untreated parasites, it cannot be concluded that the lines arose as a direct result of the U.V. treatment rather than from spontaneous mutations.

Despite the limited success of the selection experiments using U.V. irradiations, an extension of the use of mutagens in malaria studies should not be neglected. It would be possible to use those mutagens whose mechanism of action is clearly understood, to investigate the type of mutational event which gave rise to an altered parasite phenotype by carrying out reversion studies. For,

example, the mutagenic action of U.V. is known to be associated with the production of thymine-dimers in the DNA (Wacker et al., 1962). The presence of these dimers eventually leads to permanent alterations in the genetic code due to an error in replication in the portion of the DNA containing the dimer (reviewed by Deering, 1962 and Witkin, 1969). Similarly in the field of chemical mutagens, it is now known that 5-bromouracil or 2-amino-purine treatment may result in an A-T base pair in DNA being converted to a G-C pairing, and acridine dyes are thought to act by the insertion or deletion of a single nucleotide causing a frame-shift mutation (Brenner et al., 1961). If a mutagen can revert the altered parasite phenotype back to the original parent type from which it was derived, it provides information on the original genetic change resulting in the altered phenotype. For example, a variant whose genetic origin is associated with a major alteration in the arrangement of the genetic material or with a deletion or addition of a single base will not be found to have a high frequency of reversion to the original phenotype when treated with base analogues. Such a study would have to be carried out in conjunction with genetic studies to distinguish back mutations from suppressor mutations occurring in another part of the genome than that which contained the original mutation, but would provide insight into the origins of parasite variants.

(2) Characterisation Studies

(a) Drug Tests.

A number of different drug tests were investigated during a search for a test which would be sufficiently rapid to enable a single researcher to set up and monitor the large number of drug tests required for genetic work. At one stage a suppressive test based on the type devised by Thurston (1950a) was investigated. This test allowed a dose-response curve to be produced and following the method of Rollo (1952b), an ED_{50} (the effective dose which eliminates 50% of the parasite population) to be interpolated from the dose-response curve. However, a series of trial tests of this type produced rather variable results. Various statistical transformations of the results were carried out (including probit analysis) with little improvement. Statistical advice was sought and Dr W. Hill of the Institute of Animal Genetics suggested that insufficient data was obtained in each test to construct a satisfactory dose-response curve. He suggested that a statistically less suspect approach would be to use a fixed drug dose test. Accordingly the modified Warhurst drug test (Warhurst and Folwell,1968 and Peters 1968b) was selected.

A 1% parasitaemia was chosen as the end point of this drug test due to the shorter period of exponential growth of P.b.yoelii infections compared with P.b.berghei

N strain infections for which Warhurst and Folwell (1968) had used a 2% parasitaemia as the end point. The results of this test with pyrimethamine proved successful; the six drug-resistant lines obtained by selection could be clearly and repeatedly distinguished from the parent parasite lines with this test. However the test did not conclusively identify differences in the degree of resistance to pyrimethamine exhibited by the pyrimethamine-resistant lines. It is evident from the results of selection experiments which employ a gradual build up in the strength of drug pressure, that lines do exist which possess different degrees of resistance to the drug. Diggens (1970) obtained two resistant lines in her single session pyrimethamine selection experiments, one from a mouse and the other from a hamster infected with P.b.berghei strain NK65, which she reported as exhibiting similar degrees of resistance to pyrimethamine. In the present study there was some indication that the lines derived from strain 17X were less resistant to the action of pyrimethamine than the lines derived from 33X and N67. However it is possible that the selection technique adopted resulted in only lines highly resistant to pyrimethamine being obtained.

The use of the modified Warhurst drug test with sulphadiazine was not satisfactory. Variation occurred in the results obtained, both within and between experiments, which was considerably larger than that experienced

with the pyrimethamine drug tests. One explanation of the cause of this large variation could be the presence of concomitant infections of Eperythrozoon coccoides or Haemobartonella muris, which have been shown to affect the growth of malaria blood infections (Kretschmar, 1963; Peters, 1963 and 1965a; Ott and Stauber, 1967 and Büngener, 1968). However, examination of blood smears throughout the period in which this research was carried out has never revealed the presence of such infections.

An alternative explanation of this large variation is related to irregularities in the amount of PABA administered. Since the early studies of Maier and Riley (1942), and Marshall et al (1942), it has become well known that the action of sulphonamides on malaria parasites could be antagonised by PABA. Hill (1950) and Thurston (1950a and 1950b) demonstrated that the antagonism of PABA on the action of sulphadiazine extended to P.berghei infections. As the growth rates were erratic in groups of mice given identical treatments in single drug tests, it is unlikely that the large variation recorded in the results was due to the particular combination of drug and PABA used. Furthermore, Thurston (1954) when showing that the antagonism of sulphadiazine by PABA was competitive, indicated that a dose of 1.5 mg/kgm (given twice daily orally or subcutaneously) antagonised 0.5mg/kgm of sulphadiazine, but that the dose of PABA had to be increased to 50 mg/kgm to inhibit 5.0 mg/kgm of the drug.

In the present study, the highest dose of sulphadiazine used was 500 mg/kgm, while a PABA concentration of 0.005% was added to the mouse drinking water. Thurston (1954) found that a mouse weighing 20gm drinks about 4 ml of waterin 24 hours, of which approximately 3.5 ml is taken at night. The mice used in the drug tests in this present study also each weighed approximately 20 gm. It can be estimated therefore that about 0.2 mg of PABA was given daily to each mouse in conjunction with 2, 125 or 500 mg/kgm of drug. Thus at the highest drug treatments the level of PABA is unlikely to have been sufficiently large to have strongly affected the action of the drug.

It may be that the irregularities in PABA between mice were sufficient to cause the large variation in results obtained. For example, although it is known that the solid mouse diet was deficient in PABA, it may also have been subject to batch variation. Alternatively the volume of drinking water consumed by individual mice might have varied. The ranking of the results in order of apparent increase in drug-resistance did suggest that there was an apparent gradual increase in the resistance of the strains to sulphadiazine throughout the period in which the drug tests were conducted. This could have been caused by a gradual increase in the level of PABA in the mouse diet acting to reduce the effectiveness of the drug. "t" tests on the difference in the means of the period between inoculation of the

parasites and the recording of a 1% parasitaemia in C57 mice maintained with a 0.05% or a 0.005% concentration of PABA in the drinking water of the hosts were carried out to see whether such changes in the PABA could affect the test results. The "t" test carried out on the mixed strain results did suggest that the change in PABA supplement affected the parasite growth significantly, although the "t" test carried out on the difference in means of line 17X(Pr1) alone did not suggest there was a difference in growth rates of this parasite line on the two PABA concentrations. In addition, other factors such as the actual variation in the size of the estimated parasite inocula used to initiate infections might have contributed to the size of the difference in the means which was obtained.

Thurston (1954), noted that the action of pyrimethamine was also antagonised by PABA. However considerably higher doses of the antagonist were required to inhibit a certain dose of pyrimethamine than to inhibit the same dose of sulphadiazine. Although it cannot be ruled out that some factor unrelated to PABA was the cause of the variation in drug test results obtained (such as the presence of a further inhibitor, for example Hodge and Schneider (1972) found that fructose inhibits the action of sulphadiazine on bacterial infections), nevertheless the results of the tests on the dependency of parasite lines on PABA for growth also suggest that

irregularities in the PABA of the mouse diet might have been sufficient to affect the sulphadiazine drug test results. Peters (1970b and 1973) has suggested that subtle "standard" diet used in his drug test experiments with differences in the PABA content of the sulphonamides was the cause of differences in the results of repeat experiments.

The investigation of the dependence of the parasite lines on PABA for growth was undertaken because Jacobs (1964) had found that a pyrimethamine-resistant line of P.berghei had an increased requirement for PABA as compared with the parent line from which it was derived. However in the present study line 33X(Pr3) was found to require less PABA for development than the other parasite lines, although the growth of line 33X(Pr3) was not entirely independent of the presence of the PABA supplement. The comparison of the PABA requirements of the two sets of clones derived from 17X and 33X(Pr3) did indicate that within each set of cloned lines there were either parasite lines with different requirements for PABA, or more probably, that irregularities in the PABA levels of the host mice were responsible for some of the variation in the results obtained with this test.

The PABA requirement and sulphadiazine drug tests together suggest that line 33X(Pr3) differed from the other pyrimethamine-resistant lines. Jaswant Singh et al (1954b) suggested that a sulphadiazine-resistant line which was indifferent to the presence or absence of PABA, might

therefore be able to produce its own source of PABA. However, the growth of line 33X(Pr3) was not entirely independent of the presence or absence of the PABA supplement used in this study, but this could be explained by the line being able to make or obtain a proportion of its PABA from a source not available to the other resistant lines.

To obtain more reliable and repeatable results for the response to sulphadiazine and requirement of PABA for growth, it would be useful to investigate conducting drug tests in mice fed on a PABA-free solid diet and given oral injections of PABA supplements, so that the PABA content of the diets can be properly standardized.

(b) Development site

It is well known that for some rodent malarias invasion of the blood cells is restricted to reticulocytes, that is immature erythrocytes (which appear larger and stain a strong blue in Giemsa stained blood smears). There is now little doubt that red blood cell destruction in malaria infections is in excess of the level of parasitaemia, although the cause of this is uncertain. Mechanisms such as splenomelagy (George et al, 1966), osmotic fragility (Fogel et al, 1966), deterioration in erythrocytic membrane function (Dunn, 1969)

and autoimmunization (Zuckerman, 1963 and reviewed 1969) have been suggested as responsible for the destruction of uninfected erythrocytes. The red blood cell destruction stimulates the haemopoietic centres of the host and increases the production of the reticulocytes.

If the parasite line is limited to invasion of reticulocytes then the size of the parasite population is ultimately controlled by the reticulocyte response of the host (Ott et al (1967) and Ott (1968)). On the other hand, if the parasite line is not limited to development within reticulocytes, which generally form only a small proportion of the red blood cell population, its growth pattern is quite different and it usually reaches a high parasitaemia before the immune response of the host is sufficiently stimulated to eliminate the infection.

As the parasite lines in this study could be clearly seen to differ in their development site characteristics with consequential effects on virulence, it was hoped to use this characteristic as a marker in genetic crosses.

There have been reports of changes in the development site of parasite lines in the blood in association with the development of other characters. For example, Peters (1965d and 1968b) has noted with P.berghei, that increase or decrease in resistance to chloroquine is associated with a change in virulence. Similarly,

Arnold (1967) has reported that the development of resistance to chloroquine, pyrimethamine and sulphone in lines of P.berghei were also accompanied by the loss of the ability to invade mature erythrocytes. Arnold further noted that certain conditions of the photoperiodic rythmn and endocrine state also affected the ability to invade mature erythrocytes. In addition, it is well known that the virulence of rodent plasmodia tend to increase from that of the original isolate when the strain is maintained by repeated blood passage (for example, Peters, 1968a).

In the present study, 17X(Pr1), N67(Pr2), 33X(Pr4) and N67(Pr6) all retained the development site associated with the parasite line from which they were derived by selection, lines N67(Pr2) and N67(Pr6) both retaining the ability to invade mature erythrocytes unlike the pyrimethamine-resistant line reported by Arnold. However, 33X(Pr3) acquired the ability to invade mature erythrocytes on becoming resistant to pyrimethamine although 33X from which it was derived develops only in reticulocytes, and 17X(Pr5) although originally restricted to reticulocyte invasion, changed after it had failed to undergo mosquito passage for a considerable period and acquired the ability to invade mature erythrocytes as well.

(3) Crossing Experiments

Greenberg and Trembley were the first to attempt to cross lines of plasmodia (Greenberg and Trembley, 1954a and 1954b; Trembley and Greenberg, 1954, and Greenberg, 1956). To conduct a cross with lines of plasmodia it is necessary to allow the two lines to undergo cyclical passage together. As it is not possible to separate micro- and macrogametes, the products of the cross can be expected to include the parental phenotypes. To obtain conclusive proof that hybridization of the two parental lines has occurred, it is necessary firstly, to identify the presence of non-parental parasite types among the products of the cross, and secondly, to isolate these new phenotypes to make certain that complementation of the two parental phenotypes is not responsible for the new parasite type. In addition it is necessary to carry out a series of control experiments to demonstrate that the new phenotype could not have arisen by mutation or by a gene transfer mechanism other than hybridization (for example, transduction or episomal transfer).

The crosses reported by Greenberg and Trembley which yielded evidence of non-parental phenotypes among the products of the cross were between pyrimethamine-resistant and sensitive lines of P.gallinaceum. Greenberg and Trembley also reported the results of "crosses" between lines differing with respect to their growth

characteristics (Greenberg and Trembley, 1954a and Trembley and Greenberg, 1954), and on a number of occasions between lines differing in their response to pyrimethamine (Greenberg and Trembley, 1954b and Greenberg, 1956), where no evidence was obtained of non-parental phenotypes among the blood parasites arising from the joint mosquito passage.

Trembley and Greenberg (1954) mentioned the different gametocyte producing capacities of the parasite lines employed in these crosses. During the present study a number of unsuccessful "crosses" were also carried out, when although both parental parasite phenotypes emerged from the joint mosquito passage, no evidence for hybridization was obtained. From these experiments it was ascertained that a precondition for successful crosses was the use of two parasite lines which produced very heavy mosquito infections. It seems likely therefore, that the lack of hybridization experienced by Greenberg and Trembley may well have been the result of using poor gametocyte producing lines.

Turning to their more successful cross however, Greenberg and Trembley (1954b) carried out a cross between P.gallinaceum line M - a benign parasite line which was unusual in that it did not produce a secondary erythrocytic stage, and a sub-line of line BI which was pyrimethamine-resistant and virulent. A combined infection of the two lines, initiated with equal numbers

of the two types of blood parasites, was passaged twice through mosquitoes before the resulting products were analysed. The sporozoite induced infections obtained after the second joint mosquito passage was found to include pyrimethamine-resistant parasites and was associated with benign growth characteristics, that is, a non-parental phenotype.

A number of criticisms can be made of the design of this experiment. Firstly, the cross was not carried out with cloned parasite lines to ensure genetic uniformity of the parental parasites. Secondly, the mixed blood infection was allowed to develop for five days in a chick prior to conducting the cross, by which time a parasitaemia of 82% was recorded, and yet no control experiments were carried out which examined the effect of this joint passage on their growth characteristics. The mixed blood infection underwent two mosquito passages before analysis of the products of the cross was carried out and consequently, gametocytes formed in the sporozoite-induced infection derived from the first cross (including any hybrid parasites) would contribute to the gene pool of the second mosquito passage. Finally, Greenberg and Trembley used as a genetic marker a growth characteristic which they knew to be unstable (Haas et al., 1948; Trembley et al., 1951, and Greenberg, 1956). Garnham (1966) summarised the information on these American substrains of

P.gallinaceum as follows:

"when P.gallinaceum is induced to live under a variety of unnatural conditions it seems to change its morphology, to lose its capacity to produce gametocytes, to produce no malaria pigment in erythrocytic schizonts, to develop in haemopoietic cells and to grow exclusively with or without phanerozoites. Probably few of these substrains represent true mutations, because after a number of normal transmissions, most of them will regain the character of the type."

Thus in Greenberg and Trembley's experiment, after the two rounds of mosquito passage, when the virulence associated with the precross mixture had disappeared, the presence of pyrimethamine-resistance with benign growth could have arisen by a number of routes: 1) mutation of the M line confering resistance to pyrimethamine; 2) mutation or physiological change of the BI line confering benign growth characteristics, 3) hybridisation, or 4) another form of gene transfer mechanism such as transduction, "synpholia" (i.e. a type of episomal transfer) between the parental parasite lines (an opportunity for such an exchange occurred during Greenberg and Trembley's experiment during the 5 days of joint growth of the parental parasite lines in the chick used to initiate the cross). Insufficient controls were carried out to determine which of the above alternatives had been responsible for the results obtained.

Nevertheless, the "apparent transfer of pyrimethamine resistance" reported by Greenberg and Trembley (1954b) acted as a guide for later attempts to conduct crosses with malaria parasites.

Reports of crosses employing two of the pyrimethamine-resistant lines selected during the present study have now been published (Walliker *et al*, 1971 and 1973, and Oxbrow, 1972). The additional genetic marker used to analyse these crosses was glucose phosphate isomerase(GPI). In these experiments care was taken to carry out the appropriate control experiments to prove conclusively that the non-parental phenotypes obtained with the crossing technique employed, were due to hybridization, and not, for example, to an independent mutation arising during the crossing experiment among the drug-sensitive parasite line, or a gene transfer mechanism other than hybridization such as transduction, episomal transfer or synpholia. The isolation of a parasite phenotype among the products of the cross which is drug sensitive, but associated with the GPI type of the drug-resistant parent line indicates that mutation is not responsible for the appearance of new parasite phenotypes during the cross. In addition, the products of the cross were not subjected to drug pressure before cloning and therefore the high proportion of the new resistant phenotype is unlikely to have been the result of an independant mutation. Yoeli *et al*., (1969) reported a gene transfer mechanism between malaria parasites, which they termed

sympholia ("together in the nest"), which they suggested occurred between 2 parasites developing within the same blood cell. Walliker et al., (1973) carried out control experiments during the crossing studies to examine whether new parasite phenotypes were obtained during joint blood and liver development of the two parent parasite lines employed in the cross. The design of the crossing technique was believed not to be conducive for sympholia, as high parasitaemias of the joint infections did not occur, and no evidence for this phenomenon was obtained from the control studies.

In the present study a third pyrimethamine-resistant line, 33X(Pr3), was crossed with a pyrimethamine-sensitive line, 17X, and again non-parental phenotypes were discovered among the products of the cross. The expected and actual ratios of the phenotypes among the products of the cross were as follows:

	pyrimethamine-resistant GPI-1	pyrimethamine-sensitive GPI-1	pyrimethamine-resistant GPI-2	pyrimethamine-sensitive GPI-2
expected	1	3	3	1
actual	14	0	23	3

However, it is to be expected that the ratios of the different phenotypes among the products of a cross between malaria parasites will not be as informative as the ratios obtained in crossing experiments with free living organisms. Only limited control is possible of a cross conducted within mosquitoes in which both male and female gametes of each parasite line are present.

An attempt was made in this study to equalise the number of exflagellating microgametes of each line which are contributed to the blood mixture used to initiate the cross. In addition the sporozoite-induced products of the cross were stored in liquid nitrogen as soon as they became patent, to prevent distortion of the proportions of the parasite phenotypes among the cross products, due to differences in growth rates. However, the final proprtions of the parasite phenotypes among the product clones might also have depended upon differences in (i) oocyst and sporozoite maturation rates; (ii) the number of sporozoites contributing to the sporozoite yield; (iii) sporozoite viability levels, and (iv) parasite growth rates in the liver and blood stages of the parasite phenotypes. In fact lines 17X and 33X(Pr3) did differ in their growth rates and therefore this probably contributed to the deviation of the ratio of the phenotypes among the products of the cross.

Lines 33X(Pr3) and 17X also differed with respect to a number of characteristics in addition to their response to pyrimethamine, and their GPI type. These additional characteristics were, development site and dependency on PABA for growth. As was mentioned previously (page 100) the tests devised to analyse these characters did not prove entirely satisfactory. Of the 40 product clones analysed for their development site, two proved to have a variable development site, i.e. sometimes invading only reticulocytes and sometimes also

invading mature erythrocytes. The analysis of the characteristics of the product clones suggests that the response to pyrimethamine and development site did tend to segregate together. However, it also is possible that the number of blood passages between conducting the cross and analysing the products was sufficiently large to affect the development site recorded, for as mentioned previously, multi-blood passage of R development site lines is associated with the acquisition of the ability to invade mature erythrocytes.

Only 27 of the 40 product clones were analysed for their dependency on PABA for growth. The results obtained for this character with the pure line clones were more varied than had been expected from the original experiment on the uncloned parasite lines. It can be argued that the interpretation of these results are beset with similar problems as Greenberg and Trembley's results from crosses utilising growth characters, and demonstrates again the difficulties encountered with attempting to use differences in this type of character in genetic studies.

Seven clones derived from the products of a further cross, carried out between lines 17X(Pr1) and 33X were examined for their response to pyrimethamine using the modified Warhurst drug test. The purpose of this examination was to see if the levels of resistance of the product clones were different from that of the two parent lines. Although rather large variation in the results of these tests was obtained (particularly

with the 100 mg/kgm drug dose) there was no evidence of markedly different responses to pyrimethamine from those of the parent lines, among the product clones.

In the present series of crosses involving pyrimethamine-resistance conducted at Edinburgh, no evidence has been obtained for genetic transfer among the parasites by "synpholia". The crossing technique was however not conducive to the occurrence of this phenomenon, as the two parent lines involved in the cross were not grown to high parasitaemias within the same rodent. Proof of this type of gene transfer, which was first reported by Yoeli, et al., (1969), rests on establishing the frequency with which resistance appears after the selection of pyrimethamine-sensitive parasites. In the system used by Yoeli and his co-workers a pyrimethamine-resistant P.b.vinckei parasite line and a pyrimethamine-senstive P.berghei parasite line, were grown together in a single mouse until a high level of parasitaemia was obtained. Thereafter the parasites were sub-inoculated into 12 hamsters and 6 of these were later observed to contain drug-resistant, P.berghei parasites.

Diggens (1970) demonstrated that pyrimethamine-resistant P.berghei could arise after exposure of sensitive parasites to the same level of drug as used in the study of Yoeli et al. In her study, Diggens recorded that 1 out of 20 hamsters treated with drug yielded a resistant line. Although Yoeli and his co-workers give no figures for the frequency of development

of their 6 pyrimethamine-resistant lines, using the assumptions and observations concerning blood volume and erythrocyte counts obtained in this study, it is possible to estimate that more than 10^9 parasites were present in the original mixed infection at the time it was sacrificed, and that about 10^{10} parasites were subjected to drug treatment in the hamsters. It is suggested from the results of selection experiments reported in this study that a population of 10^{10} parasites is quite likely to contain a pyrimethamine-resistant mutant of spontaneous origin, and although the presence of six independant mutants in this size of population is highly improbable, the design of the experiment would allow all the resistant parasites to have originated from one resistant clone.

In conclusion therefore, it will be seen that the evidence reported by Yoeli et al (1969) for the occurrence of sympholia is not entirely convincing. To demonstrate conclusively the occurrence of a gene transfer phenomenon such as sympholia, frequencies of development of resistance must be calculated.

(4) Future Developments in Genetic Studies

Genetic studies of plasmodia can be expected to make significant contributions to malaria research at a number of different levels. A study of enzyme variation has already been suggested will be of use in supplementing and refining the morphological and physio-

logical criteria by which taxonomic division is made (Carter, 1973). This type of information should lead to a clearer understanding of the relationships between the human malaria parasite species, and the reasons underlying the distribution of malaria strains throughout the world.

It is also important to determine the genetic variation of experimental parasite material currently held in laboratories. Some of these parasite strains will have accumulated extensive variation during the long periods since their isolation from the wild. Uniformity of parasite populations is of great importance in many fields of malaria research. For example, the clearest results of host antibody and parasite antigen interaction will inevitably come from studies where the simplest, that is genetically uniform, antigenic stimulus is presented to the host immune-response system.

It must be hoped that new techniques will become available to allow the investigation into the mutational or adaptation origins of malaria variants, such as drug-resistance and virulence, to continue. In addition, a useful approach to investigate the molecular changes underlying a mutant character might come from reversion studies with mutagens (see page 97). At present an obstacle to the study of molecular changes associated with drug-resistance using this approach is the absence of a technique to isolate sensitive parasites from a predominantly drug-resistant parasite population, such

as the replica plating methods available to bacteriologists, other than the time consuming method of cloning and testing of parasite lines. In the present work an attempt was made to develop such a technique by using differences in PABA requirements of pyrimethamine-resistant and sensitive lines. Although this preliminary study was unsuccessful, it might be possible to develop this method by using plasmodial strains with very marked differences in PABA requirements and by preparing a solid mouse diet completely free of PABA.

Dihydrofolate reductases isolated from chick liver have been examined by electrophoresis on starch and polyacrylamide gels (Huennekens et al., 1971). It might therefore be profitable to continue the study of the different types of pyrimethamine-resistant parasites obtained by examining the dihydrofolate reductases of pyrimethamine-resistant and sensitive parasites by electrophoretic techniques. An illustration of the type of information which genetic studies on drug-resistant parasite lines might produce is afforded by the results of Hutchinson (1971) on aminopterin-resistant bacteria. Aminopterin is an antifolate in the same class as pyrimethamine. In studies on Diplococcus pneumoniae, three different genetic classes of mutant have been isolated. These classes are; a common transport group in which genetic change results in reduced binding of the drug to the carrier component; a rarer class in which the amount of dihydrofolate reductase present is increased

or the affinity of dihydrofolate reductase for the drug is altered, and a third class in which resistance is due to a different, unknown mechanism.

In higher organisms it is possible to locate a gross chromosomal alteration resulting in a phenotypic variant by cytological examination of the karyotype. In malaria parasites, however, there is still confusion as to the number of chromosomes in individual cells (Wolcott, 1954 & 1957); Bano, 1959; Canning & Anwar, 1968 & 1969; Howells & Davies, 1971 and Canning and Sinden 1973) and in view of the difficulties of examining these chromosomes, it seems most unlikely that cytological studies will be able to contribute to the study of the genetic origins of specific variants such as pyrimethamine-resistant parasites.

The location of gene changes has been studied in other organisms, and drug-resistance has in some cases been found to be determined by cytoplasmic factors. Some classes of erythromycin-resistance in yeast (Thomas and Wilkie, 1968, and Cohen et al., 1970) and in Paramecium aurelia (Beale, 1969 and Beale et al., 1972) have been found to be determined by cytoplasmic genetic factors located in the mitochondria. In bacteria, classes of drug-resistance have been shown to be associated with episomes or R factors which are cytoplasmic (Watanabe and Fukasawa, 1961a, and Watanabe, 1963). For studies on the location of genes it is necessary to carry

out reciprocal matings so that the influence of the cytoplasm, which is largely contributed by the macrogamete can be investigated. Thus to commence the study of the location of genes in plasmodia a technique is needed to separate micro- and macrogametes. However, when bacteria are exposed to the action of intercalating dyes such as ethidium, acriflavine or acridines, the R factors are degraded (Watanabe and Fukasawa, 1961b, and Mitsuhasi et al., 1961) and if the R factors were carrying drug-resistance then the bacteria revert to drug sensitivity. Yeast cells are also susceptible to these agents which act by stopping mitochondrial synthesis and degrading pre-existing mitochondrial DNA (Goldring et al., 1970, and Perlman and Mahler, 1971). As the mitochondria of rodent plasmodia are not active during the trophozoite stages of the life cycle of the parasite (Howells et al., 1969 and Howells, 1970) it is possible that the use of intercalating agents might prove useful in the study of gene location in malaria.

Other types of gene transfer should also be investigated further. Transduction, the phage mediated transfer of small sections of the bacteria genome from one bacterium to another, is caused by the production of a defective virus particle containing a few bacterial genes (reviewed by Hayes, 1964). Virus particles have been found in plasmodia (Terzakis, 1969), but as yet no investigation of this type of gene transfer operating between plasmodia has been made. In addition the

studies on synpholia could be extended which would require calculating frequencies of the development of resistant lines under the conditions most conducive for this type of transfer.

In summary, a number of different problems could benefit from genetic studies. Genetic studies on malaria should, however, provide valuable information on the extent of the parasite's genetic potential for change.

APPENDIX A

The Standard PABA Supplement to the Host Diet

(1) Adoption of a 0.05% PABA supplement to the drinking water of the host mice, as a standard addition to their laboratory diet.

During the initial stages of this research programme, an attempt was made to identify a suitable technique for assessing parasite drug response for use in conjunction with genetic studies.

A difficulty encountered in this work was the presence of a considerable amount of variation in the course of blood infections. For example, it was not uncommon to inoculate 10 C57 mice each with 10^6 parasites (by the intraperitoneal route in 50% v/v calf serum and Ringer's solution) and observe that while in 8 of these mice, infections became patent 1 to 2 days post-inoculum, the remaining two mice did not show circulating parasitaemias until 6 to 10 days post-inoculum. On occasions when smaller inocula were used, for example 10^4 parasitised erythrocytes, one or two of the 10 mice inoculated failed to become infected up to 21 days post-inoculum, when the experiment was discontinued.

Members of the department of Parasitology at the Liverpool School of Tropical Medicine suggested that

this type of result might be due to a PABA (p-amino benzoic acid) deficiency in the solid mouse diet used in the laboratory, and drew attention to the importance of this food additive for plasmodial growth, as suggested by the results of Hawking (1953).

At that time the laboratory mice at Edinburgh were maintained on a locally prepared rat cake called QUAYSIDE produced by McGregors of Leith. This was supplied to the mice ad libitum as was tap water.

Peters (1967) had increased the growth of P.chabaudi infections by supplementing their diet with PABA administered in the drinking water. In April 1970, the effects of various concentrations of PABA (added to the drinking water of the mice) on the course of P.b.yoelii blood infections was investigated. This was carried out as follows: 5 groups of 6 C57, 4-5 week old male mice were caged separately. Each group was assigned to one of 5 different concentrations of PABA, namely; zero, 0.0005%, 0.005%, 0.05% and 0.4%. The mice were also supplied with QUAYSIDE. Twenty four hours after commencing their PABA diets, the mice were each inoculated with 5×10^6 parasites (P.b.yoelii, line 17X). The inoculations were administered by the intraperitoneal route, in 50% v/v calf serum in Ringer's solution. The parasites inoculated had been harvested from mice maintained without a PABA supplement. On days 2, 4 and 8 post-inoculum, tail blood smears were prepared from each mouse and an estimation of parasitaemia made

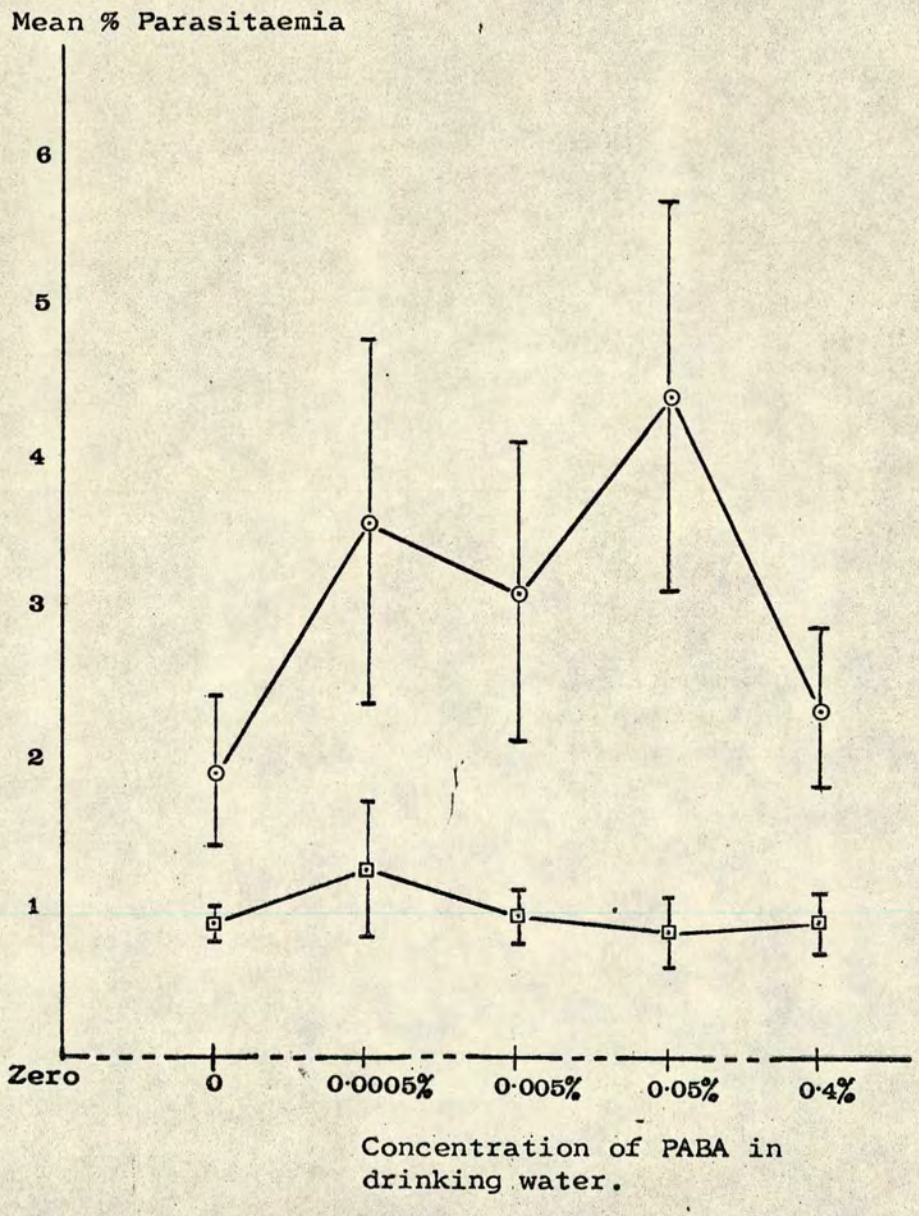
Fig.10. Growth of Infections of Line 17X in mice maintained on different PABA supplements.

Mean % parasitaemia of infections in 6 C57 mice ± 2 SE against PABA concentration (for results of individual mice see Table 24, Appendix B).

Key

⊡ mean % parasitaemia on day 4 post inoculum

⊙ mean % parasitaemia on day 8 post inoculum



(materials and methods, page 20). The mean results recorded for each group of mice are presented in Table 19 and figure 10, while individual mouse results are included in Table 30, Appendix B.

As Table 19 demonstrates, line 17X exhibited higher mean parasitaemias on day 8 in all groups of mice maintained on PABA supplemented drinking water, than in the group of mice receiving tap water alone. The intermediate concentrations of PABA, namely 0.05% and 0.005%, were associated with the highest mean parasitaemias, whereas the 0.4% solution appeared to be less efficient at promoting parasite growth. In fact, excessive amounts of PABA (4 to 8%) in the diet have been reported to cause suppression of parasitaemia with P.berghei infections (in rats - Hawking and Terry, 1957 and in mice - Kretschmar, 1965) and with P.vinckei infections (in hamsters - Adler and Foner, 1961).

Subsequently, Mr A. Oxbrow, also of the Protozoan Genetics Unit at Edinburgh University, carried out a series of experiments comparing the growth of parasite infections maintained on one of three diets, namely:
i) QUAYSIDE solid diet with a 0.05% solution of PABA in the drinking water;
ii) Dixon's 41B solid diet (used for malaria research at the Liverpool School of Tropical Medicine) either without a PABA supplement or
iii) with a 0.05% concentration of PABA in the drinking water. His results showed no significant differences

Table 19. Mean Parasitaemia of groups of mice maintained on varying concentrations of PABA

Parasite strain	No. of Parasites inoculated day 0 by intrapentoneal route	Concentration of PABA in the drinking water	Estimates of Parasitaemia ± S.E. Mean results of each group of 6 mice Day post-inoculum:-		
			2	4	8
17X	5×10^6	zero	0.05 ± 0.02	0.88 ± 0.05	1.87 ± 0.26
		0.0005%	0.08 ± 0.03	1.25 ± 0.22	3.55 ± 0.60
		0.005%	0.10 ± 0.02	0.95 ± 0.09	3.08 ± 0.50
		0.05%	0.12 ± 0.02	0.84 ± 0.11	4.37 ± 0.64
		0.4%	0.07 ± 0.02	0.90 ± 0.11	2.30 ± 0.26

The individual parasitaemias exhibited by mice in this experiment on days 2, 4 and 8 post-inoculum are included in Table 30. Appendix B.

in parasite growth in mice maintained on any of these diets (Oxbrow 1970: unpublished results).

It was decided therefore to standardly maintain all laboratory mice on a 0.05% solution of PABA in tap water, in conjunction with the QUAYSIDE solid mouse diet. Mice were placed on this diet as soon as they entered the laboratory, and at least 7 days were allowed to elapse before they were inoculated with parasites.

This regime has proved satisfactory and is used by all the workers in the Edinburgh laboratory for the standard maintenance of strains. However, on occasions an infection in one mouse in a group inoculated with a standardized inoculum size has been found to become patent several days after the rest of the group. It is considered that this could be due to faulty inoculation technique host variation or PABA factors. The rather low parasitaemias recorded on day 8 post-inoculum in the original experiment to study the effect of PABA on the growth of line 17X have, in subsequent passages increased, presumably as a result of continually maintaining the line with a PABA supplement.

It has never proved necessary to supplement the diet of Grammomys surdaster with PABA. These rodents were also maintained on QUAYSIDE, and with tap water supplied ad libitum with a constant supply of pieces of fresh apple.

(2) Parasite growth with a 0.05% solution of PABA supplementing the QUAYSIDE solid mouse diet.

For a detailed comparison of the growth rates of different parasite lines, it would be necessary to grow all the lines simultaneously in the laboratory to minimise batch variation of the solid food. This was not carried out with the parasite lines used in this research programme after April 1970 when it became customary to maintain all laboratory mice on a 0.05% PABA supplement.

To obtain exactly comparable results for a series of parasite lines it would also be necessary to standardise the recent laboratory histories of these lines, as it is well known that such factors as the number of blood passages since mosquito transmission would affect the growth characteristics of a particular line. However continued handling of the 9 parasite lines used in this study allowed their growth characteristics, after recent mosquito transmission to be separated into two distinct types: i) those lines which grow in mature and immature erythrocytes and which generally kill their hosts and

ii) those lines which usually infect reticulocytes and which even if they do eventually reach moderately high levels of parasitaemia rarely kill their hosts.

The day on which the first type of parasite line kills its host or the second reaches its maximum parasitaemia will depend upon such factors as the strain and

age of the mice and the state of development of their immunological responses, the size of the original parasite inoculum and in the case of some infections on the proportions of various types of red blood cells in the host's blood.

An illustration of these two types of parasite growth characteristics are given in Table 20. These results are derived from one experiment when 10^6 parasites of either line 33X or 33X(Pr3) were inoculated in 50% v/v calf serum in Ringer's solution by the intraperitoneal route on day 0, into 5 week old, male C57 mice.

Examples of initial growth patterns of P.b.yoelii and P.b. nigeriensis parasite infections in mice receiving a 0.05% PABA supplement (i.e. the period between inoculation and the recording of a 1% parasitaemia have been included in Table 21 in view of the importance of this part of the parasite growth pattern for modified Warhurst drug tests.

Table 20. % Parasitaemia in 5 week old C57 male mice infected with either line 33X or line 33X(Pr3) and maintained with a 0.05% solution of PABA in the drinking water.

Parasite line	Number of parasitized erythrocytes inoculated Day 0	Mouse Number	Parasitaemia Day Post-inoculum																			
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
33X	10^6	1	0.04	0.70	3.8	1.5	1.6	2.5	4.9	4.4	10	16	18	23	54		36	30					killed
		2	0.04	0.70	2.1	1.7	1.1	4.5	5.1	5.9	12	14	15	32	28		45	20					"
		3	0.02	0.11	6.3	5.8	3.5	5.0	10	20	30	36	22	60	55		51	23					"
		4	0.00	0.01	0.8	2.2	1.0	0.8	1.5	4.0	5.1	8.0	7.5	6.0	25		23	59					"
		5	0.00	0.00	0.7	1.0	0.9	1.0	2.0	3.5	4.6	8.0	5.1	12	17		27	23					"
33X (Pr3)	10^6	1	0.00	0.00	0.00	0.02	0.4	3.9	8.0	12	35	39	58	died									
		2	0.00	0.00	0.08	3.5	11	29	30	57	died												
		3	0.00	0.08	0.9	6.5	9.6	34	58	74	died												
		4	0.02	0.50	3.4	15	24	25	39	died													

Table 21. Initial Growth Patterns of P.b.yoelii & P.b. nigeriensis Parasite infections initiated with 10^6 parasites on day 0, by the intraperitoneal route.

Parasite line	Mouse host	Date of inoculation	Mouse No.	% Parasitaemias Day post-inoculum:- 1	2	3	4	5
33X	C57	28.7.71	1	0.15	2.1			
			2	0.17	1.6			
			3	0.33	2.0			
			4	0.36	2.4			
33X(Pr3)	C57	28.7.71	1	1.12	2.1			
			2	0.29	2.3			
			3	0.10	2.0			
			4	0.24	1.4			
			5	0.02	0.28	1.8		
33X(Pr4)	C57	28.7.71	1	1.13	2.1			
			2	0.12	2.4			
			3	0.08	0.74	1.3		
			4	0.13	0.54	0.80	1.9	
17X	TO	10.8.71	1	0.15	1.1			
			2	0.15	1.9			
			3	0.11	1.9			
			4	-	0.09	0.68	1.2	
N67	TO	10.8.71	1	0.01	0.30	3.2		
			2	-	0.70	11.3		
			3	0.01	0.55	3.2		
			4	0.04	0.59	7.8		
33X	TO	10.8.71	1	0.06	0.54	1.9		
			2	-	0.66	2.4		
			3	0.08	0.26	1.8		
			4	0.08	2.86	4.3		
17X(Pr1)	TO	10.8.71	1	0.11	1.6			
			2	-	0.09	2.08		
			3	0.04	0.36	1.30		
			4	0.05	1.3			
N67(Pr2)	TO	10.8.71	1	0.02	0.35	3.9		
			2	0.03	0.94	5.9		
			3	-	0.20	2.8		
			4	0.04	0.08	1.8		

Parasite line	Mouse host	Date of inoculation	Mouse No.	% Parasitaemias Day post-inoculum:- 1	2	3	4	5
33X(Pr3)	TO	10.8.71	1	0.02	1.1			
			2	0.05	0.62	5.4		
			3	-	0.33	4.9		
			4	0.01	1.3			
33X(Pr4)	TO	10.8.71	1	0.02	0.48	5.4		
			2	0.07	0.35	2.7		
			3	0.03	1.7			
			4	0.08	0.06	5.2		
17X	TO	18.10.71	1	-	2.3			
			2	0.05	1.6			
			3	0.01	0.34	3.3		
			4	0.01	0.15	2.2		
N67	TO	18.10.71	1	0.03	0.07	1.0		
			2	0.08	0.32	4.9		
			3	0.01	1.9			
			4	0.04	0.05	1.5		
33X	TO	18.10.71	1	0.03	0.33	3.5		
			2	0.04	1.07			
			3	0.01	1.7			
			4	0.04	1.0			
17X(Pr1)	TO	18.10.71	1	-	-	0.12	5.0	
			2	0.02	1.7			
			3	-	0.5	5.7		
			4	0.01	1.6			
33X(Pr3)	TO	18.10.71	1	0.05	2.2			
			2	0.06	1.6			
			3	0.05	2.0			
			4	0.08	2.6			
17X	TO	21.3.72	1	0.6	0.6	0.7	0.39	1.6
			2	0.3	0.83	3.5		
			3	-	0.11	1.4		
			4	0.09	1.0			
N67	TO	21.3.72	1	0.05	0.87	6.0		
			2	-	0.50	0.22	2.6	
			3	-	0.03	2.5		
			4	-	0.32	7.3		
33X	TO	21.3.72	1	0.01	0.14	1.7		
			2	0.02	0.65	1.0		
			3	0.08	0.76	4.8		
			4	0.01	0.36	0.66	1.1	

Parasite line	Mouse host	Date of inoculation	Mouse No.	% Parasitaemia Day post-inoculum:- 1	2	3	4	5
17X(Pr1)	TO	21.3.72	1	-	0.01	0.08	2.4	
			2	0.02	1.2			
			3	0.03	1.0			
			4	0.02	0.67	2.5		
33X(Pr3)	TO	21.3.72	1	0.11	1.0			
			2	0.06	1.0			
			3	0.04	1.7			
			4	0.07	1.2			
33X(Pr3)	TO	21.3.72	1	0.02	0.59	1.8		
			2	0.01	0.73	4.0		
			3	0.05	0.56	2.3		
			4	0.03	0.34	5.0		
17X	TO	2.4.72	1	0.06	1.9			
			2	0.05	3.3			
			3	0.04	1.8			
			4	0.06	2.5			
17X(Pr1)	TO	2.4.72	1	0.10	1.3			
			2	0.04	1.0			
			3	0.02	2.3			
			4	0.01	1.4			
33X(Pr3)	TO	2.4.72	1	0.02	0.62	4.6		
			2	-	0.48	7.8		
			3	0.06	0.79	9.0		
			4	-	0.31	3.2		
33X(Pr4)	TO	2.4.72	1	0.02	0.30	4.4		
			2	-	0.16	3.8		
			3	-	0.05	1.1		
			4	-	0.42	3.6		
N67(Pr6)	TO	2.4.72	1	0.05	2.9			
			2	0.08	2.2			
			3	0.09	1.3			
			4	0.07	3.4			
N67	TO	21.8.72	1	0.05	1.3			
			2	-	-	0.14	3.1	
			3	0.01	1.5			
			4	0.13	2.0			
N67(Pr2)	TO	21.8.72	1	0.03	0.73	9.0		
			2	0.05	0.39	5.7		
			3	0.01	0.23	3.9		
			4	0.04	0.80	7.3		

Parasite line	Mouse host	Date of inoculation	Mouse No.	% Parasitaemia Day post-inoculum:- 1	2	3	4	5
33X	TO	21.8.72	1	0.02	1.5			
			2	0.01	1.3			
			3	-	0.32	2.8		
			4	-	0.30	2.5		
17X(Pr5)	TO	21.8.72	1	0.17	3.7			
			2	0.06	5.4			
			3	0.05	3.5			
			4	0.14	3.4			

APPENDIX B

Tables of individual mouse results

Table 22. The effect of Ultraviolet Irradiation on parasite viability. (These results were used to prepare Fig.4, the Dose Response Curve).

The time taken to reach a 1% parasitaemia by individual T.O. Mice inoculated with aliquots of a concentrated parasitized erythrocyte suspension (line 33X) which had been treated with zero, 560, 1680, 2800, 5000 or 7000 ergs/mm^2 of U.V.

Dose of U.V. ($ergs/mm^2$)	Time between inoculation and the recording of a 1% parasitaemia:-				
	Individual Mice Results 1	2	3	4	Mean result ±SE
zero	1.4	1.0	1.6	1.4	1.4 ± 0.25
560	2.0	2.7	1.8	1.9	2.1 ± 0.20
1680	2.0	2.8	2.1	2.8	2.4 ± 0.22
2800	2.4	2.2	2.5	2.0	2.3 ± 0.11
5000	2.9	3.4	2.9	2.6	3.0 ± 0.17
7000	5.4	6.0	5.0	5.3	5.4 ± 0.21

Table 23a[1] Response to Pyrimethamine

The duration of 'test periods'[2] in individual infected mice receiving zero (controls), 2, 50 and 75 mg/kgm of pyrimethamine

Parasite line	Experiment Number[4]	Date of Parasite Inoculation[3]	Duration of 'Test Period' Dose of Pyrimethamine (mg/kgm) 0	2	50	75
17X	6	11.8.71	2.0	5.0	8.5	
			1.8	5.3	7.9	
			1.8	4.8	9.0	
			3.8	4.0	8.1	
	7	18.10.71	1.6	5.3	7.5	
			1.7	3.1	7.1	
			2.3	4.8	7.4	
			2.5	2.4	7.3	
	8	21.3.72	2.0	3.5	8.6	9.8
			2.8	3.3	7.5	9.0
			1.8	5.6	7.9	10.3
			2.2	4.5	8.1	(5)
	9	17.8.72	1.8	3.9	5.9	7.9
			1.7	5.5	5.9	6.9
			1.8	3.9	5.7	7.7
			1.7	2.7	5.6	7.6
N67	2	1.6.71	2.6	6.9		
			2.7	6.7		
			2.9	6.6		
			3.4	7.1		
	6	11.8.71	2.5	5.0	7.0	
			2.1	6.1	7.6	
			2.4	5.7	8.4	
			2.2	6.0	6.8	

cont..

1. The above table is an expanded version of Tables 9a and 9b respectively. In the latter tables only mean results of each treatment group are recorded.

2. For a definition of the 'test period' see Materials and Methods section page 33.

3. Each mouse was inoculated with 10^6 parasites, by the intraperitoneal route, on this day.

4. Experiments 1-5 were carried out in C57 mice, whereas experiments 6-9 were carried out in mice of the TO strain.

5. In a number of cases insufficient mice were available for each drug treatment to be repeated in 4 mice. It was usual on these occasions to allocate only 3 mice to the group receiving the largest drug dose.

N67 cont.	7a	18.10.71	2.2	4.7	6.6	
			1.8	7.0	7.4	
			2.4	4.6	5.8	
			2.1	4.7	7.1	
	7b	18.10.71	2.0	3.5	6.8	
			1.6	4.6	5.0	
			1.5	3.1	6.3	
			1.8	3.4	4.3	
	8	21.3.72	2.0	3.5	7.6	7.9
			3.6	3.0	6.7	7.1
			2.3	4.0	7.7	7.7
			1.9	2.7	7.6	6.9
	9	17.8.72	1.9	3.4	6.4	6.8
			1.9	3.8	6.0	7.7
			1.7	3.9	5.6	7.6
			2.0	3.8	5.8	6.8
33X	5	28.7.71	1.8	6.0	10.0	
			1.9	4.6	7.7	
			1.6	2.5	8.0	
			1.6	3.6	8.5	
	6	11.8.71	2.5	5.0	8.4	
			3.3	5.0	7.5	
			2.6	6.0	8.4	
			1.7	5.1	8.2	
	7	18.10.71	2.3	5.8	6.6	
			1.8	5.3	8.0	
			1.7	5.6	7.4	
			1.9	4.5	6.7	
	8	21.3.71	2.7	6.0	7.8	9.6
			2.9	5.7	7.8	9.9
			2.1	2.9	7.6	7.6
			3.8	4.6	8.2	8.8
	9	17.8.72	1.9	6.0	7.3	10.2
			2.0	5.0	8.3	11.9
			2.5	4.1	11.7	9.7
			2.6	4.8	10.5	10.8
17X(Pr1)	1	25.5.71	2.9	2.1	6.8	
			3.0	3.8	5.8	
			2.9	3.0	6.6	
			1.0	2.7	7.4	
	3	16.6.71	2.5	2.8		
			2.6	3.0		
			2.0	2.9		
			2.2	3.0		

cont...

17X(Pr1) cont..	4	29.6.71	1.8	2.0		
			1.8	2.6		
			2.0	1.9		
			2.0	2.3		
	6	11.8.71	1.9	1.9	4.8	
			2.8	1.9	4.9	
			2.9	2.9	5.6	
			1.9	3.4	(5)	
	7	18.10.71	3.4	2.2	4.1	
			1.6	1.7	2.2	
			2.4	1.7	3.2	
			1.6	1.7	2.2	
	8	21.3.72	1.9	3.0	3.5	5.6
			3.2	1.9	3.3	5.9
			2.6	1.9	4.8	6.8
			2.5	2.0	5.6	5.6
	9	17.8.72	2.0	1.9	2.4	5.2
			2.0	1.8	2.7	5.3
			1.8	1.8	2.8	5.8
			1.9	1.9	3.8	4.2
N67(Pr2)	3	16.6.71	1.8	2.0		
			1.9	2.1		
			3.6	1.8		
			1.8	1.9		
	4	29.6.71	2.1	1.9		
			2.2	1.9		
			2.1	2.1		
			1.9	2.3		
	6	11.8.71	2.8	2.1	3.3	
			2.6	2.1	3.0	
			2.5	2.4	3.1	
			2.0	2.4	2.7	
	9	17.8.72	2.1	2.5	2.4	2.8
			2.4	2.1	2.4	3.2
			2.5	3.3	2.5	3.5
			2.3	3.1	2.5	2.9
33X(Pr3)	5	28.7.71	1.9	2.9	5.9	
			2.3	2.3	6.0	
			2.5	4.0	6.6	
			3.9	3.9	6.1	
	6	11.8.71	2.3	2.0	3.7	
			2.5	2.6	5.5	
			1.8	2.0	3.7	
			2.2	2.1	2.8	

cont...

33X(Pr3)7 cont..		18.10.71	1.4	1.7	1.7	
			1.6	1.6	1.9	
			1.6	1.6	1.7	
			1.5	1.6	1.8	
	8	21.3.72	1.9	1.7	1.7	2.1
			1.9	2.3	2.3	3.8
			1.8	2.1	2.1	2.2
			1.8	3.3	(5)	2.4
	8	21.3.72	2.4	2.5	2.7	2.9
			2.3	2.3	3.3	3.1
			2.1	1.9	3.9	3.1
			2.4	2.1	3.0	4.6
	9	17.8.72	2.2	2.1	3.1	3.8
			2.4	2.2	4.5	4.8
			2.1	2.3	2.5	4.5
			2.5	2.2	2.5	(5)
33X(Pr4)	6	11.8.81	2.0	2.0	4.0	
			2.2	2.3	3.1	
			2.4	2.2	3.2	
			2.1	2.2	2.9	
	9	17.8.72	2.5	2.7	3.3	3.9
			2.6	2.7	3.0	5.0
			2.9	2.6	2.9	4.4
			2.4	2.6	2.8	4.1
17X(Pr5)	9	17.8.72	1.6	1.9	2.9	5.0
			1.6	1.8	2.9	5.5
			1.7	1.7	3.4	4.8
			1.6	1.8	3.8	5.5
N67(Pr6)	9	17.8.72	1.8	1.9	2.4	3.2
			1.8	2.1	2.2	3.4
			1.9	1.7	2.3	3.5
			1.7	1.7	2.2	3.2

Table 23b. Tabular presentation of results in Fig.5. Summation of results from individual drug tests for each parasite line (for the methods of calculating mean increase in 'test period' and standard errors, see Appendix C). SE = standard error. Time in Days.

Parasite line	No. of experiments carried out	Dose of Pyrimethamine given (mg/kgm):- 2		50		75	
		Mean Increase in 'Test period' ± SE	Limits of ± 2 SE	Mean increase in 'Test period' ± SE	Limits of ± 2 SE	Mean increase in 'Test period' ± SE	Limits of ± 2 SE
17X	4	2.14 ± 0.25	1.64 - 2.64	5.29 ± 0.22	4.85 - 5.73	6.51 ± 0.39	5.73 - 7.29
33X	6	2.69 ± 0.24	2.21 - 3.17	5.76 ± 0.33	5.10 - 6.42	7.25 ± 0.54	6.17 - 8.33
N67	5	2.23 ± 0.25	1.73 - 2.73	4.53 ± 0.18	4.17 - 4.89	5.15 ± 0.18	4.79 - 5.51
17X(Pr1)	7	0.67 ± 0.18	0.31 - 1.03	1.69 ± 0.26	1.17 - 2.21	3.31 ± 0.21	2.89 - 3.73
N67(Pr2)	4	0.64 ± 0.24	0.16 - 1.12	0.34 ± 0.10	0.14 - 0.54	0.78 ± 0.16	0.46 - 1.10
33X(Pr3)	6	0.29 ± 0.12	0.05 - 0.53	0.87 ± 0.20	0.47 - 1.27	1.25 ± 0.26	0.73 - 1.77
33X(Pr4)	2	0.05 ± 0.02	0.01 - 0.09	0.76 ± 0.18	0.40 - 1.12	1.75 ± 0.24	1.27 - 2.23
17X(Pr5)	1	0.18 ± 0.04	0.10 - 0.26	1.62 ± 0.22	1.18 - 2.06	3.58 ± 0.18	3.22 - 3.94
N67(Pr6)	1	0.20 ± 0.10	0.00 - 0.40	0.48 ± 0.05	0.38 - 0.58	1.53 ± 0.07	1.39 - 1.67

Table 24. Comparison of the effect of PABA on the growth of nine parasite lines.

Parasitaemias on day 4 post-inoculum in 2 groups of mice, 1 group being maintained with a PABA supplement (0.05% solution) in their drinking water.

Parasite line	Addition to Host's drinking water	Estimate of Parasitaemia Day 4 post inoculum. Mouse Number:- 1	2	3	4
17X	PABA	4.9	4.1	6.0	3.1
	NONE	0.2	0.8	0.7	0.2
N67	PABA	55	38	23	60
	NONE	7.5	0.5	0.0	0.0
33X	PABA	6.3	5.8	7.4	2.3
	NONE	0.0	2.1	0.01	0.0
17X(Pr1)	PABA	3.8	6.7	4.4	7.9
	NONE	0.0	0.0	0.0	0.0
N67(Pr2)	PABA	32	33	42	39
	NONE	0.0	7.7	6.7	0.3
33X(Pr3)	PABA	65	58	44	49
	NONE	0.03	0.4	5.5	0.2
33X(Pr4)	PABA	6.4	8.5	12.5	10.9
	NONE	0.0	0.0	0.1	2.0
17X(Pr5)	PABA	14	7.0	4.3	13
	NONE	2.8	0.01	0.1	0.7
N67(Pr6)	PABA	65	58	44	49
	NONE	0.03	0.4	0.2	5.5

Table 25[1] Response to Sulphadiazine

The duration of 'test periods'[2] in individual infected mice receiving zero (controls), 25, 125 and 500 mg/kgm of sulphadiazine.

Parasite Line	Experiment No.	Date of Parasite inoculation[3]	Duration of 'TEST PERIOD' Dose of Sulphadiazine (mg/kgm) 0	25	125	500
17X	2	21.3.72	3.2	2.4	4.0	3.9
			1.8	2.7	3.5	3.4
			2.6	1.9	4.2	2.4
			1.8	3.0	3.8	2.5
	3	15.5.72	3.7	6.6	7.5	10.9
			4.6	5.9	7.6	10.6
			3.8	5.7	8.1	8.6
			3.2	6.0	8.1	8.9
	6	17.8.72	2.6	6.0	9.7	10.4
			1.9	6.7	10.1	10.7
			1.8	5.6	9.8	11.5
			2.6	6.2	10.3	12.7
N67	3	15.5.72	2.9	3.1	4.4	8.6
			3.5	4.4	4.5	8.7
			2.3	5.6	4.0	11.6
			2.6	4.9	4.6	4.5
	4	3.8.72	3.0	4.6	9.9	12.0
			3.8	4.5	11.9	11.2
			2.3	7.7	10.2	11.9
			3.0	7.7	11.0	12.8
33X	2	21.3.72	2.8	2.9	5.7	7.0
			2.0	4.1	7.0	9.0
			2.5	2.7	8.2	9.8
			3.0	3.0	5.9	8.4
	5	9.8.72	2.3	2.9	6.0	4.7
			1.8	3.3	4.7	5.8
			3.5	3.7	5.4	7.3
			2.7	4.7	5.2	6.4

1. The above table is an expanded version of Table 11, page 67; in the latter table only mean results of each treatment group are recorded.

2. For a definition of the 'test period' see Materials and Methods section page 33.

3. Each mouse was inoculated with an estimated 10^6 parasites by the intraperitoneal route on this day.

4. In this experiment insufficient mice were available for each drug treatment to be repeated in 4 mice. Therefore only 3 mice were allocated to the group of mice receiving the largest drug dose.

Table 25 continued

17X(Pr1)	1	23.2.72	3.2	3.7	8.0	9.0
			3.3	4.1	7.0	8.0
			3.0	4.7	7.9	7.9
			3.5	4.0	6.6	7.9
	2	21.3.72	4.1	3.8	5.8	4.7
			3.6	4.2	4.8	5.4
			3.1	3.6	6.0	5.5
			3.0	3.5	5.0	(4)
	6	17.8.72	2.8	5.4	8.8	
			2.7	4.9	9.6	
			2.8	4.8	9.8	
			2.8	5.9	9.3	
N67(Pr2)	3	15.5.72	2.8	4.6	4.8	4.9
			2.7	3.9	4.0	5.6
			2.5	4.3	4.6	5.9
			2.2	3.9	4.7	7.6
	4	3.8.72	3.8	4.6	10.9	11.5
			3.9	4.5	13.3	12.5
			3.7	3.7	11.3	11.3
			3.6	4.8	10.3	11.4
33X(Pr3)	1	23.2.72	2.2	2.9	3.3	3.4
			2.4	3.2	3.2	3.4
			2.7	3.0	2.9	3.5
			2.2	2.6	3.3	3.3
	2	21.3.72	1.8	1.8	1.9	2.5
			2.0	2.2	3.8	2.4
			1.8	2.0	2.5	3.0
			1.9	2.2	1.9	2.3
	4	3.8.72	3.4	4.1	5.2	6.4
			3.2	4.4	4.8	5.8
			3.0	4.0	3.6	5.6
			3.2	4.7	4.9	4.6
33X(Pr4)	5	9.8.72	2.9	4.9	6.2	6.9
			2.6	4.2	6.7	6.8
			2.4	5.0	6.7	5.9
			2.3	5.1	5.5	6.0
17X(Pr5)	6	17.8.72	2.8	5.1	7.0	7.7
			1.9	6.0	6.6	7.6
			2.8	4.8	6.7	8.6
			1.9	5.7	6.9	(4)

cont...

Table 25 cont...

N67(Pr6)	3	15.5.72	2.4	4.0	4.5	5.0
			3.0	4.0	4.8	7.6
			3.3	3.9	4.3	6.0
			3.0	4.3	3.8	5.9
	4	3.8.72	4.7	3.8	3.1	6.2
			3.0	6.0	4.9	6.1
			1.9	6.6	5.8	7.5
			3.0	5.2	5.8	7.4

Table 26a. Response to Pyrimethamine of 7 clones derived from the products of a cross between line 17X(Prl) and line N67.

The duration of "test periods"(2) in individual, male, T.O. infected mice receiving zero (controls), 2, 50 and 100 mg/kgm of pyrimethamine.

Parasite Clone Number	Date of Parasite Inoculation(3)	Duration of "test period" Dose of pyrimethamine (mg/kgm):- 0	2	50	100
1	2.3.72	2.0	2.1	6.1	6.5
		1.8	1.8	6.3	8.2
		1.6	2.3	6.4	7.8
		1.6	2.3	6.1	7.0
2	2.3.72	2.1	2.6	5.7	6.9
		2.6	2.7	5.8	6.5
		3.1	4.0	5.8	6.4
		2.1	2.7	5.8	7.0
3	2.3.72	1.9	1.5	2.7	5.5
		1.7	1.5	3.3	3.8
		1.5	1.4	2.7	3.1
		1.7	1.6	2.8	3.4
4	2.3.72	1.7	1.7	3.1	3.6
		1.8	1.7	2.5	3.3
		1.9	1.6	3.0	4.2
		1.8	1.8	(4)	5.8
5	2.3.72	1.7	2.2	6.4	5.8
		2.2	3.5	5.6	6.2
		1.6	3.8	6.3	5.3
		2.0	3.1	6.0	6.1
6	2.3.72	3.0	1.8	4.0	4.4
		1.6	1.8	3.3	4.2
		2.1	2.0	4.3	4.7
		2.2	3.2	4.3	4.3
7	2.3.72	2.2	1.7	3.1	3.6
		1.8	1.5	3.4	4.4
		1.7	1.7	2.9	4.7
		1.6	1.8	3.3	4.3

1. The above table is an expanded version of Table 18, page 85. In the latter table only mean results of each treatment group of mice are recorded.

2. For a definition of the 'test period' see Materials and Methods Section, page 33 .

cont....

Table 26a cont...

3. Each mouse was inoculated with an estimated 10^6 parasites by the intraperitoneal route on this day.

4. In this experiment insufficient mice were available for each drug treatment to be repeated in 4 mice. Therefore only 3 mice were allocated to the group of mice receiving the largest drug dose.

Table 26b. Tabular presentation of results in Fig.9.

Response to pyrimethamine of 7 clones derived from the products of a cross between lines 17X(Pr1) and N67.

Clone No.	Drug Dose (mg/kgm)	Increase in the 'test period' in comparison with controls ± SE	Limits of ± 2SE
1	2	0.3 ± 0.16	0.0 - 0.6
	50	4.4 ± 0.12	4.2 - 4.6
	100	5.6 ± 0.40	4.8 - 6.4
2	2	0.5 ± 0.41	-0.3 - 1.3
	50	3.3 ± 0.24	2.8 - 3.8
	100	4.2 ± 0.28	3.6 - 4.8
3	2	0.2 ± 0.09	0.0 - 0.2
	50	1.2 ± 0.16	0.9 - 1.5
	100	2.3 ± 0.55	1.2 - 3.4
4	2	0.1 ± 0.06	0.0 - 0.4
	50	1.1 ± 0.18	0.7 - 1.5
	100	2.4 ± 0.56	1.3 - 3.5
5	2	1.3 ± 0.38	0.5 - 2.1
	50	4.2 ± 0.23	3.7 - 4.7
	100	4.0 ± 0.25	3.5 - 4.5
6	2	0.0 ± 0.45	-0.9 - 0.9
	50	1.8 ± 0.38	1.0 - 2.6
	100	2.2 ± 0.30	1.6 - 2.8
7	2	-0.1 ± 0.14	-0.4 - 0.2
	50	1.4 ± 0.17	1.1 - 1.7
	100	2.4 ± 0.27	1.9 - 2.9

Table 27. The characteristics of 20 clones derived from line 17X

Clone Number	Development type	GPI type	Effect of PABA on parasite growth: % Parasitaemia day 4 post inoculum NO PABA Mouse number 1	2	3	0.05% PABA Mouse number 1	2	3	Ratio*
1	R	1	0.75	0.40	0.0	1.9	2.0	3.2	16
2	R	1	0.0	0.06	0.07	1.8	1.6	1.7	2
3	R	1	0.85	1.0	0.01	1.9	4.7	5.5	16
4	R	1	0.32	0.30	0.0	2.3	5.4	4.2	5
5	R	1	0.01	0.02	0.12	3.4	3.0	3.0	2
6	R	1	1.0	0.07	0.09	3.2	3.6	2.3	12
7	R	1	1.0	1.4	0.03	2.3	7.7	2.9	19
8	R	1	1.2	0.01	0.0	4.6	3.9	2.6	19
9	R	1	0.22	0.01	0.08	3.2	2.4	3.9	3
10	R	1	0.03	0.0	0.0	3.4	3.2	3.6	0
11	R	1	0.06	0.12	0.05	5.2	9.8	3.0	13
12	R	1	0.15	0.03	0.04	6.6	5.2	5.2	12
13	R	1	0.05	0.24	0.09	10.1	5.0	3.0	2
15	R	1	0.02	0.42	0.05	1.8	3.7	5.3	5
16	R	1	0.35	0.01	0.05	3.7	2.9	4.7	3
17	R	1	0.07	0.13	0.02	3.2	3.8	3.3	2
18	R	1	0.0	0.24	0.04	4.3	2.9	1.3	10
19	R	1	0.0	0.07	0.01	2.6	1.7	3.2	6
20	R	1	0.0	0.05	0.09	2.3	2.8	4.2	2

* Ratio = $\frac{\text{mean parasitaemia day 4 post inoculum without PABA}}{\text{mean parasitaemia day 4 post inoculum with a 0.05\% PABA supplement}} \times 100$

Table 28. The characteristics of 20 clones derived from line 33X(Pr3)

Clone Number	Development type	GPI-type	Effect of PABA on Growth: % Parasitaemia day 4 post inoculum						Ratio*
			NO PABA Mouse number			0.05% PABA Mouse number			
			1	2	3	1	2	3	
1	M&R	2	2.0	33	38	53	38	45	54
2	M&R	2	14	28	1.6	17	22	37	54
3	M&R	2	23	13	32	31	17	25	96
4	M&R	2	3.5	6.5	31	37	22	15	56
5	M&R	2	20	11	13	22	17	28	67
6	M&R	2	24	7.3	15	34	25	52	41
7	M&R	2	23	24	14	45	26	27	62
8	M&R	2	17	21	5.8	41	58	13	38
9	M&R	2	9.6	9.9	30	17	36	15	74
10	M&R	2	3.2	21	4.3	35	32	12	66
11	M&R	2	8.2	60	14	38	48	46	66
12	M&R	2	7.7	6.8	4.3	32	16	2.7	36
13	M&R	2	3.3	39	8.0	18	43	68	39
14	M&R	2	13	35	23	60	66	50	40
15	M&R	2	7.7	24	34	36	5.9	46	76
16	M&R	2	6.4	20	32	31	53	42	45
17	M&R	2	65	13	6.8	50	24	62	45
18	M&R	2	7.0	7.2	40	13	30	25	65
19	M&R	2	5.1	20	8.0	14	19	34	50
20	M&R	2	12	24	22	52	41	64	34

*Ratio = $\frac{\text{mean parasitaemia day 4 post-inoculum without PABA}}{\text{mean parasitaemia day 4 post-inoculum with a 0.05\% PABA supplement}} \times 100$

Table 29. Characteristics of 40 clones derived from the products of a cross between lines 17X and 33X(Pr3).

Clone Number	Sensitive or Resistant	Development type	GPI type	Effect of PABA on parasite growth: % parasitaemia Day 4 post inoculum						*Ratio
				NO PABA Mouse number			0.05% PABA Mouse number			
				1	2	3	1	2	3	
1	Res	M&R	1	12	0.4	16	38	30	48	24
2	Res	M&R	1	4.6	2.0	0.27	19	18	17	13
3	Res	M&R	2	20	22	6.7	21	14	16	94
4	Res	M&R	2							
5	Res	M&R	2							
6	Res	M&R	2							
7	Res	M&R	2							
8	Res	M&R	2	10	8.8	8.7	31	63	9.0	28
9	Res	M&R	2							
10	Res	M&R	1	3.8	7.2	3.7	11	15	14	33
11	Res	M&R	2	0.06	0.0	0.01	19	45	51	0
12	Res	M&R	2							
13	Res	M&R	1	16	0.52	0.78	9	10	12	56
14	Res	M&R	1	2.0	6.3	8.0	13	14	12	54
15	Res	M&R	1	0.09	1.4	0.20	4.4	20	15	3
16	Res	M&R	2	2.6	39	20	52	40	12	61
17	Res	M&R	2							
18	Res	M&R	2							
19	Res	M&R	2							
20	Res	R	1	7.9	1.0	5.0	2.9	2.9	12	78
21	Sens	R	2	0.46	0.50	0.40	6.0	5.1	8.0	9
22	Res	M&R	1	1.9	6.4	1.1	17	21	12	22
23	Res	M&R	2							
24	Res	M&R	2	16	4.3	20	0.6	23	25	79
25	Res	R	1	0.0	0.0	0.0	5.9	3.6	14	0
26	Res	M&R	2	6.7	12.6	6.2	19.6	36	25	32
27	Res	M&R	2	3.2	2.7	3.0	0.0	5.0	16	27
28	Res	M&R	1	0.0	0.44	0.08	05	10	19	1
29	Res	V	1	3.1	0.05	0.14	5.2	15	6	13
30	Res	M&R	2	15	11	3.2	33	24	41	30
31	Sens	R	2							
32	Res	M&R	1	10	7.0	7.3	3.5	32	45	22
33	Res	M&R	2							
34	Res	R	2							
35	Res	M&R	2	1.8	4.4	18.	28	15	15	42
36	Res	M&R	2	4.1	4.7	10	14	19	12	42
37	Res	M&R	2	2.8	4.1	1.3	44	5.9	8.0	45
38	Res	M&R	2	6.0	3.5	3.6	14	6.5	4.8	53
39	Res	V	1	7.8	11	4.0	38	14	17	33
40	Sens	R	2	4.9	0.68	1.1	2.6	3.3	2.5	73

$$*\text{Ratio} = \frac{\%\text{ parasitaemia day 4 post-inoculum without PABA}}{\%\text{ parasitaemia day 4 post-inoculum with a 0.05\% PABA supplement}} \times 100$$

Table 30. Parasitaemias recorded in mice maintained with varying concentrations of PABA added to their drinking water.

(Parasite line is *P.b.yoelii*, 17X; infections initiated with an inoculum size of 5×10^6 parasites; for further details of the experiment see Appendix A).

Concentration of PABA in drinking water	Mouse Number	Estimates of Parasitaemia Individual results from 6 infections in mice. Day post-inoculum:-		
		2	4	8
0	1	0.07	0.97	1.5
	2	0.01	0.83	1.4
	3	0.02	0.88	2.6
	4	0.07	0.67	2.7
	5	0.04	0.91	1.2
	6	0.12	1.02	1.8
0.0005%	1	0.05	1.90	3.8
	2	0.20	1.28	3.1
	3	0.06	1.74	5.6
	4	0.02	1.14	3.8
	5	0.07	1.09	3.9
	6	0.10	0.37	1.1
0.005%	1	0.10	0.81	5.3
	2	0.09	1.24	2.0
	3	0.16	0.62	3.6
	4	0.16	0.97	2.2
	5	0.08	0.95	2.4
	6	0.02	1.10	3.0
0.05%	1	0.21	1.34	6.6
	2	0.17	0.82	3.6
	3	0.09	0.90	2.2
	4	0.10	0.77	5.6
	5	0.06	0.61	4.4
	6	0.07	0.63	3.8
0.4%	1	0.03	0.71	2.1
	2	0.03	0.66	1.8
	3	0.12	1.03	3.0
	4	0.07	0.71	2.8
	5	0.05	1.36	2.7
	6	0.12	0.91	1.4

APPENDIX C

1. Comparison of Two Samples of Unequal size using Student's 't' test.

(The 't' test formula was obtained from the statistics course Notes of Dr R.C.Roberts of the Institute of Animal Genetics, Edinburgh University. The 't' test was developed by Gosset (1908).)

The question asked in this test is "could both samples be random samples drawn from the same population?"

To obtain a measure of the difference to be expected from the means of samples drawn at random from the same population it is necessary to calculate the sampling variance of a difference.

Thus, if the means of the two samples to be compared are $\bar{X}_1$ and $\bar{X}_2$

then $$V_{(\bar{X}_1 - \bar{X}_2)} = V_{\bar{X}_1} + V_{\bar{X}_2}$$

$$= \frac{\sigma^2}{n_1} + \frac{\sigma^2}{n_2}$$

where σ^2 = the population variance

n_1 n_2 are the sizes of the two samples.

An estimate of the population variance can be obtained from the 2 original samples

$$V = \frac{\sum x_1^2}{(n_1-1)} + \frac{\sum x_2^2}{(n_2-1)}$$

$$= \frac{x_1^2 + x_2^2}{n_1 + n_2 - 2}$$

where x_1^2, and x_2^2 are the "sums of squares" of the two original samples.

Substituting this estimate of the population variance into the previous formula

$$V_{(\bar{X}_1 - \bar{X}_2)} = V\left(\frac{1}{n_1} + \frac{1}{n_2}\right)$$

$$= \frac{\sum x_1^2 + \sum x_2^2}{n_1 + n_2 - 2} \left(\frac{1}{n_1} + \frac{1}{n_2}\right)$$

$$t(n_1 + n_2 - 2) = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{V_{(\bar{X}_1 - \bar{X}_2)}}}$$

where the subscript to t is the appropriate degrees of freedom.

"t" tests carried out

1. The time taken by all the parasite lines to reach a 1% end point in the modified Warhurst drug tests in C57 mice, on a 0.05% PABA supplement (Table 23) has been compared with the time taken by the parasite lines to reach this end point in modified Warhurst drug tests in C57 mice on a 0.005% PABA supplement (Table 25). The comparison was made between the growth rates of "control" mice which did not receive drug treatment.

Results of the "t" test:-

	Sample 1	Sample 2
PABA supplement:	0.005%	0.05%
means of samples: $\bar{X}_1$ and $\bar{X}_2$	2.98	2.37
"sums of squares": $\sum x_1^2$ and $\sum x_2^2$	638.40	177.32
number in samples: n_1 and n_2	72	32

$t = 2.63$

$df = 102$

$p = 1\%$

Therefore, only once in a 100 times would the difference observed between the sample means be expected to arise by chance if both samples had been drawn from the same population.

2. To avoid comparing the growth rates of a mixture of lines, a "t" test was carried out on the results of

drug tests with 17X(Pr1). This parasite line was chosen as it was the one with which the largest number of drug tests had been carried out in C57 mice. Again the individual results of the infections on 0.05 and 0.005% PABA supplements, in C57 mice not receiving drug treatment which constituted the two samples for comparison are to be found in Tables 23 and 25, in Appendix B. Results of the "t" test:

	Sample 1	Sample 2
PABA supplement	0.005%	0.05%
means of samples: $\bar{X}_1$ and $\bar{X}_2$	3.16	2.26
"sums of squares": $\sum x_1^2$ and $\sum x_2^2$	121.57	63.15
numbers in samples: n_1 and n_2	12	12

$t = 0.23$

$df = 22$

$p = > 10\%$

Therefore more than once in 10 times would one expect to obtain a difference of this size in the sample means by chance alone.

2. Mean increase in "test periods" for individual parasite lines: pooling of experimental results.

To pool the results obtained from a number of repeated drug tests, the mean time between inoculation of parasites and recording of a 1% parasitaemia by the control (undrugged) mice was subtracted from each mouse result in the drug treated groups within the same

experiment. These adjusted results were then pooled between experiments for each drug treatment and the standard errors of the mean calculated as usual:-

$$\text{standard error} = \sqrt{\frac{v}{n}}$$

where v = variance

n = number in sample.

3. <u>The calculation of standard errors attached to the increase in "test period" within individual experiments</u>.

The calculation of the standard errors attached to the increase in "test periods" within experiments was carried out by summing the standard errors attached to the mean "test periods" of control (undrugged) and each drug-treated group of mice, as follows:-

$$\text{standard error}_1 = \sqrt{\frac{v_1}{n_1} + \frac{v_2}{n_2}}$$

where

standard error_1 = standard error attached to increase in "test period".

v_1 and n_1 = variance of "test period" results, and number of mice in control (undrugged) group.

v_2 and n_2 = variance of "test period" results, and number of mice in a drug-treated group.

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I wish to certify that apart from the assistance listed above, all the experiments recorded in this thesis were planned and carried out by myself.

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PUBLICATIONS

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Effect of PABA and sulphadiazine on two pyrimethamine-resistant *Plasmodium berghei yoelii* lines. Trans. R. Soc. trop. Med. Hyg. **66**, 542.

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Effect of PABA and Sulphadiazine on two pyrimethamine-resistant *Plasmodium berghei yoelii* lines

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Two lines of *P.b.yoelii* originating from strains 17X and 33X have been made resistant to pyrimethamine using the single-step method of Diggens (1970).

Strain 33X was irradiated with 5000 ergs/mm^2 U.V. prior to the selection procedure.

The resistant lines are termed 17X(Pr1) and 33X(Pr3).

Like other pyrimethamine-resistant lines (Jacobs 1964), line 17X(Pr1) has a slightly increased dependence on p-aminobenzoic acid (PABA) for its growth when compared to the sensitive parent strain. The growth rate of line 33X(Pr3) was found to be independent of PABA Supplementation.

Unlike other *P.berghei* lines selected for resistance to pyrimethamine (Peters 1970), line 33X(Pr3) was found to be cross resistant to sulphadiazine.

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Genetic Recombination in Malaria Parasites

THE complexity of the life cycle of malaria parasites and the lack of suitable strain characters which could be used as genetic markers have made genetic studies of these organisms difficult. We have now available, however, a number of strains of *Plasmodium berghei* isolated from wild rodents and mosquitoes in Africa, which can be differentiated from one another by starch-gel electrophoresis of certain enzymes[1]. We have also derived parasite lines resistant to the drug pyrimethamine from some of these strains, and such resistant lines remain stable even in the absence of the drug. By making crosses between lines which differ in both enzyme type and drug sensitivity, we have been able to obtain parasites exhibiting recombinant characters.

The parasite lines selected for use are described here as lines A and C. Line A was derived from strain 17X of *P.b. yoelii* and is pyrimethamine resistant. The resistance was produced in a single step[2] by treating mice infected with strain 17X with pyrimethamine at doses of 50 mg/kg daily for 4 days. Line A, like strain 17X, contains the electrophoretic form of the enzyme glucose phosphate isomerase denoted GPI-1[1]. Line C was derived from strain 33X of *P.b. yoelii*, and is pyrimethamine-sensitive. In the 4 day suppressive test[3], it is more than fifty times as sensitive to pyrimethamine as line A. Line C contains the enzyme form GPI-2[1].

The sequence in which this work was conducted is presented in Fig. 1. Equal amounts of blood (0.3 ml.) were taken from two tree rats (*Grammomys surdaster*), one of which was infected with line A and the other with line C. The blood from each animal was mixed and injected intravenously into an uninfected tree rat. This produced an immediate patent parasitaemia in the recipient (113 A + C in Fig. 1). Mosquitoes (*Anopheles stephensi*) were fed immediately on this animal in the hope that gametes of each strain would mix. The resulting sporozoites were used to infect further tree rats from which ten C57 black mice (127 A + C in Fig. 1) were subsequently infected. Five of these mice were treated with subcutaneous doses of pyrimethamine[4] at 15 mg/kg daily for 4 days. All ten mice became infected and examination of the parasites on starch gels (Fig. 2) revealed the presence of both GPI-1 and GPI-2 in all animals.

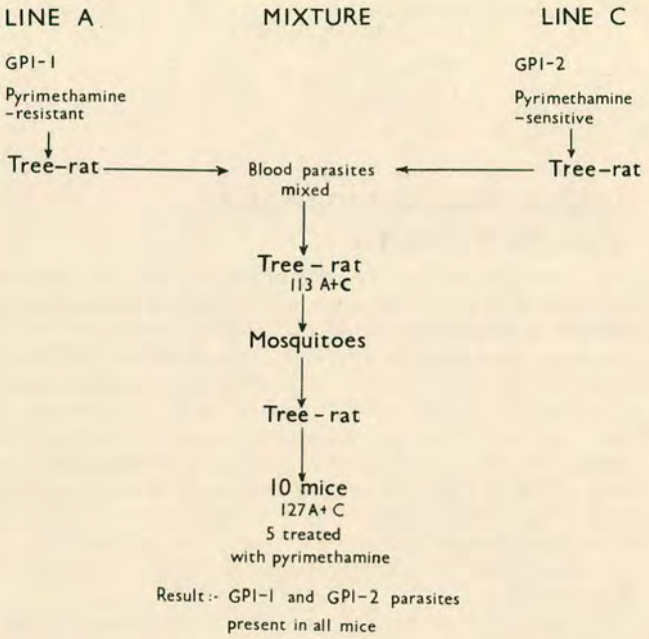

Fig. 1 Procedure adopted in producing recombinant parasites.

To confirm that the GPI-2 parasites in the treated mice were drug-resistant, infected blood from these mice was diluted in serum Ringer[5] into aliquots containing an average of one parasitized cell. The aliquots were inoculated into individual mice, from which blood infections were established. Two GPI-1 and two GPI-2 lines were derived in this way, each of which was found to grow equally well when subjected to pyrimethamine treatment.

As controls the parent lines A and C were passaged through mosquitoes, tree rats and mice in the same way as the mixture. Line A parasites survived drug treatment, and line C parasites were eradicated. The work has been repeated several times with similar results; line C has been exposed to pyrimethamine at doses of 15 mg/kg in fifty mice, none of which has remained infected. We think it very unlikely, therefore, that mutation could account for the appearance of resistant GPI-2 parasites in the mixed infections.

Two further controls were set up to determine whether the recombinant parasites obtained were due to the phenomenon described by Yoeli *et al.*[4] as "synpholia". In synpholia, it is suggested that pyrimethamine resistance can be transferred from one strain to another when they grow up together in blood

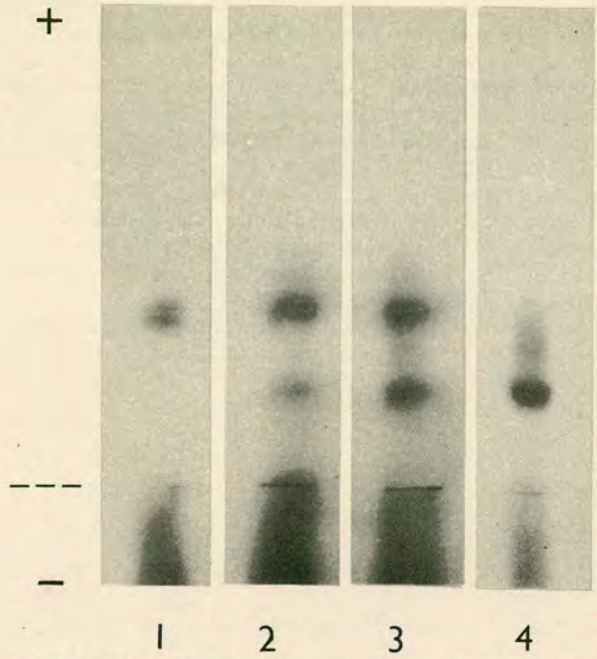

Fig. 2 Starch gel zymograms of glucose isomerase (GPI) in line A, line C, and mixtures of lines A and C. 1, Line A (GPI-1); 2, mixture following pyrimethamine treatment of mice 127A+C (showing GPI-1 and GPI-2); 3, mixture in untreated mice 127A+C (showing GPI-1 and GPI-2); 4, line C (GPI-2).

cells of the same host. In the first control, tree rat 113A+C (inoculated with equal numbers of blood parasites of each line) was kept alive for 7 days after mosquitoes had fed on it. Blood parasites from this animal were then used to infect ten mice, five of which were treated with pyrimethamine. In these mice, only GPI-1 parasites survived, but in the untreated mice both GPI-1 and GPI-2 parasites were present. In the second control, sporozoites of each parent line were pooled and inoculated into tree rats. After five days, blood parasites from these animals were used to infect ten mice, five of which were given pyrimethamine. In untreated animals, both GPI-1 and GPI-2 parasites were detected, while the animals given pyrimethamine contained only GPI-1 parasites after treatment.

We have been unable, therefore, to produce parasites exhibiting recombinant characters by simply mixing blood parasites or sporozoites of the parent lines. This does not disprove the phenomenon of synpholia, which may occur as a rare event detectable only when double selection pressure is applied[4], but suggests that it cannot account for our results.

We consider that these results are important in two respects. First, they show that a conventional genetic analysis of malaria parasites is possible. This should enable us to obtain much more precise information than has been available hitherto on the biological basis of such important characters as drug resistance and virulence. Second, they indicate that genetic recombination is continually taking place in natural populations of malaria parasites, which enables the rapid evolution of new strains and their spread in populations of animals and man. We propose to exploit the technique outlined here to investigate the nature of other types of drug resistance as well as enzyme polymorphism and virulence.

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